Biochem. J. (1993) **289**, 277–282 (Printed in Great Britain)

Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets

Trevor R. WALKER* and Steve P. WATSON

Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, U.K.

The aim of this study was to establish further the role of protein kinase C in aggregation and secretion of 5-hydroxytryptamine (5-HT) from human platelets by using the selective inhibitor Ro 31-8220. Ro 31-8220 (3 μM) inhibited completely phosphorylation of pleckstrin, the major protein kinase C substrate, induced by thrombin, A23187 or phorbol dibutyrate (PDBu). Myosin light-chain phosphorylation induced by PDBu was also inhibited completely, but that induced by thrombin or A23187 was only inhibited partially. As myosin light chain is a substrate for both myosin light-chain kinase and protein kinase C, these results suggest that Ro 31-8220 is inhibiting only the protein kinase C-induced phosphorylation and that Ro 31-8220 has a greater selectivity to protein kinase C than does its structural analogue staurosporine. The stimulation of secretion of 5-HT by maximally effective concentrations of thrombin and A23187 was decreased significantly by 3 μM Ro 31-8220, but not inhibited completely. These results indicate a major role for protein kinase C in the stimulation of secretion by agonist- and ionophore-induced activation. On its own, a maximal concentration of PDBu induced a small degree of secretion (3.3 ± 1.0 %), but potentiated markedly the response to a submaximal concentration of A23187 (300 nM) to a level greater than seen with a maximal concentration of A23187. A similar set of results was also seen with aggregation, but not with shape change. We interpret these results to mean that the signalling event for secretion and aggregation is Ca^{2+}, and this is potentiated markedly by protein kinase C. In the case of secretion, it appears that it is the synergy which is the major determining factor in influencing the extent.

INTRODUCTION

Human platelet activation by physiological agonists such as thrombin is associated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the subsequent production of the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (Nishizuka, 1984). Inositol 1,4,5-trisphosphate is released into the cytoplasm and causes Ca^{2+} mobilization from the dense tubular system, leading to activation of myosin light-chain kinase, a Ca^{2+}/calmodulin-dependent kinase, whose major substrate is myosin light-chain (20 kDa) (Daniel and Adelstein, 1976). Diacylglycerol remains in the plasma membrane and causes the activation of protein kinase C (Sano et al., 1983), the major substrate of which in platelets is a 47 kDa protein termed pleckstrin (Imaoka et al., 1983; Tyers et al., 1988). As a consequence of the generation of these two second messengers, many proteins change their phosphorylation state in association with platelet aggregation and secretion responses (Siess, 1989).

The role of protein kinase C in aggregation and secretion from human platelets has not been fully elucidated, and so the development of selective cell-permeant inhibitors is of considerable interest. Protein kinase C is composed of two domains, a regulatory domain which contains binding sites for phorbol esters/diacylglycerol and a catalytic domain which contains the ATP-binding site (Stabel and Parker, 1991). Staurosporine, which has been extensively investigated in platelets (Watson et al., 1988; Watson and Hambleton, 1989), is known to inhibit protein kinase C through the ATP-binding site, but is a relatively non-specific inhibitor of protein kinases (Tamaoki et al., 1986). Several analogues of staurosporine were synthesized in order to produce a more selective inhibitor of protein kinase C. Davis et al. (1989) reported several analogues of staurosporine which were more selective against protein kinase C in both isolated enzyme assays and cellular assay systems. In the present study, the effect of one of these analogues (Ro 31-8220) has been investigated, both to examine its selectivity of protein kinase C in platelets and to elucidate further the role of the enzyme in the activation of platelets.

METHODS

Blood was drawn on the day of the experiment from aspirin-free volunteers with sterile 20 mM sodium citrate as anti-coagulant, and 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 to increase intracellular cyclic AMP. Platelets were resuspended in 1 ml of modified Tyrode/Hepes solution (composition in mM: NaCl 138, NaH_2PO_4 0.36, KCl 2.9, $NaHCO_3$ 12, Hepes 20, glucose 5, EGTA 1, $MgCl_2$ 1; pH 7.3) and labelled with [^{32}P]P_i (2 mCi/ml for 1 h) or [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) (5 μCi/10 ml for 1 h). Platelets were then centrifuged in the presence of prostacyclin at 1000 ***g*** for 10 min and resuspended at a concentration of $(2–8) \times 10^8$ cells/ml in the above buffer, containing indomethacin (10 μM) to inhibit cyclo-oxygenase. EGTA was omitted in studies of aggregation. Platelets were left at least 30 min before experimentation.

Platelet suspensions (0.49 ml) were prewarmed at 37 °C for 5 min before addition of compounds as appropriate. Experiments were stopped by transfer to 0.5 ml of 6 % (v/v) glutaraldehyde in phosphate buffer (pH 7.3) for analyses of 5-HT secretion or to Laemmli (1970) buffer for analyses of protein phosphorylation. Protein phosphorylation and 5-HT secretion were measured as described previously (Nunn and Watson, 1987), and tryptic

Abbreviations used: PDBu, phorbol 12,13-dibutyrate; 5-HT, 5-hydroxytryptamine.

* To whom correspondence should be addressed.

peptide mapping was carried out as described by Cleveland et al. (1977).

Materials

[^{32}P]P_i (8500–9120 Ci/mmol) and [^{3}H]5-HT (21.0 Ci/mmol) were from N.E.N.–Du Pont (U.K.) (Stevenage, Herts., U.K.). Thrombin and phorbol 12,13-dibutyrate (PDBu) were from Sigma Chemical Co. (Poole, Dorset, U.K.). Prostacyclin was kindly donated by Wellcome Laboratories (Beckenham, Kent, U.K.). Ro 31-8220 was a gift from Roche Products (Welwyn Garden City, Herts., U.K.). All other reagents were of analytical grade.

Statistical analysis

Experiments were performed in duplicate, results being expressed as means ± S.E.M. of 3–6 experiments. Statistical tests were by Student's t test, and statistical significance was achieved with 95% confidence limits.

RESULTS

Effect of Ro 31-8220 on protein phosphorylation

Platelets contain a protein of apparent molecular mass 47 kDa, termed pleckstrin, of unknown function, which is the major substrate for protein kinase C (Tyers et al., 1988); phosphorylation of this protein after activation of platelets correlates closely with 5-HT secretion. Phosphorylation of pleckstrin by thrombin (1 unit/ml) and A23187 (3 μM) was inhibited completely by 3 μM Ro 31-8220, a maximally effective concentration, from 336 ± 15 and 212 ± 20% of basal to 114 ± 8 and 76 ± 6% of basal respectively ($P < 0.05$) (Figure 1a). Inhibition of stimulus-induced pleckstrin phosphorylation by Ro 31-8220 was dose-dependent (Figure 1a). The phorbol ester PDBu (300 nM) exhibited a similar increase in pleckstrin phosphorylation to that induced by thrombin, which was also inhibited completely by Ro 31-8220 (3 μM), from 345 ± 39 to 90 ± 6% of basal levels ($P < 0.05$) (Figure 1a). A23187 induced a sub-maximal increase in phosphorylation of pleckstrin in the presence of a concentration of indomethacin (10 μM) which completely inhibits endoperoxide formation (Watson et al., 1986), supporting a previous report which postulated that Ca^{2+} mobilization primes protein kinase C (Siess and Lapetina, 1988).

The other major protein which undergoes phosphorylation upon platelet activation by thrombin is myosin light chain. This is a substrate for both Ca^{2+}/calmodulin-dependent myosin light chain kinase and protein kinase C, although the action of each kinase occurs at distinct sites (Naka et al., 1983). Phosphorylation of myosin light chain induced by thrombin and A23187 is inhibited by Ro 31-8220 over a similar concentration range to that at which inhibition of pleckstrin phosphorylation is achieved. At a maximally effective concentration (3 μM), Ro 31-8220 inhibited myosin light-chain phosphorylation induced by thrombin and A23187 from 232 ± 16 and 179 ± 12% of basal to 159 ± 15 and 132 ± 9% of basal respectively ($P < 0.05$) (Figure 1b), all measurements being taken 60 s after agonist challenge. Phosphorylation of myosin light chain induced by PDBu is inhibited to below basal levels by Ro 31-8220 (3 μM), from 160 ± 10% to 95 ± 11% ($P < 0.05$) (Figure 1b). These results suggest that myosin light chain undergoes phosphorylation by myosin light-chain kinase and protein kinase C after stimulation with thrombin and A23187, Ro 31-8220 selectively inhibiting protein kinase C-mediated phosphorylation.

Tryptic-digest analysis of myosin light chain from thrombin-stimulated platelets identified four main phosphorylated fragments (Figure 2), two of which were identified in platelets pretreated with PDBu (results not shown). Phosphorylation of the co-migrating fragments was inhibited by over 90% by 3 μM Ro 31-8220 (Figure 2); the two other fragments were also seen in platelets treated with A23187 (results not shown). Phosphorylation of one of these was unaltered by Ro 31-8220, whereas the phosphorylation of the other was decreased by ~ 40% (fragments 2 and 4 respectively in Figure 2). Fragment 3 appears to be phosphorylated only by protein kinase C, whereas fragment 4 is phosphorylated by myosin light-chain kinase and protein kinase C. If the phosphorylation of fragment 3 by myosin light-chain kinase brings about an alteration in its mobility, then this may explain the absence of fragment 4 in PDBu-stimulated platelets. If this is the case, then these results provide further evidence that Ro 31-8220 selectively inhibits protein kinase C relative to myosin light-chain kinase.

The ability of Ro 31-8220 to inhibit selectively protein kinase C-mediated phosphorylation induced by thrombin was compared

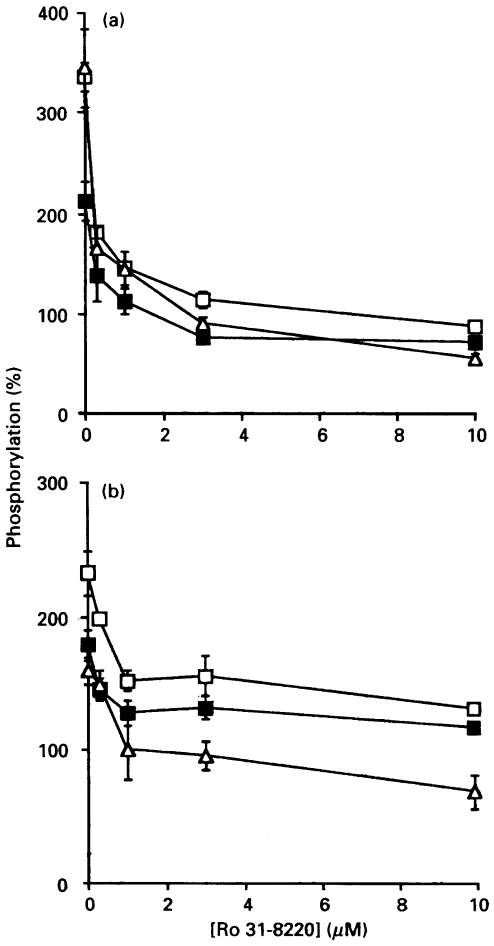

Figure 1. Effect of Ro 31-8220 (a) on pleckstrin phosphorylation and (b) on myosin light-chain phosphorylation

(**a**) Platelets were prelabelled with [^{32}P]P_i and incubated with Ro 31-8220 for 1 min at various concentrations as indicated. Phosphorylation of pleckstrin was localized by autoradiography after polyacrylamide-gel electrophoresis (11% gels), excised from the gel and counted for radioactivity in a liquid-scintillation counter. Platelets were stimulated with thrombin (1 unit/ml) (□), A23187 (1 μM) (■) or PDBu (300 nM) (△) for 1 min after preincubation with Ro 31-8220 as appropriate. (**b**) Platelets were prepared and stimulated as for (**a**). Results are expressed as means ± S.E.M. ($n = 3$–5) in (**a**) and (**b**).

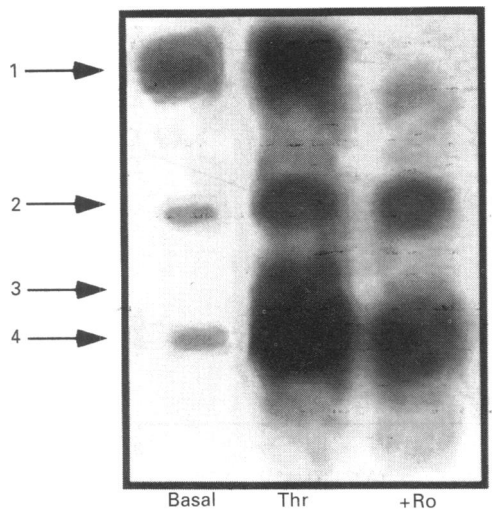

Figure 2 Tryptic peptide mapping of myosin light chain

Platelets were prepared as for Figure 1, and were stimulated with thrombin (1 unit/ml) for 1 min in the presence (+Ro) or absence (Thr) of Ro 31-8220 (3 μM), which had been preincubated for 1 min, as indicated. Myosin light chains were localized by autoradiography, excised from the gel and subjected to proteolysis by overlaying the gel slice with trypsin (1 mg/ml). The protein was run into the stacking gel and the power turned off as the dye front neared the end of the stacking gel. Power was turned on after 30 min and electrophoresis carried out as described previously for Figure 1. Subsequent fragments were separated by electrophoresis and localized by autoradiography; fragments (1–4) were labelled in terms of decreasing molecular mass.

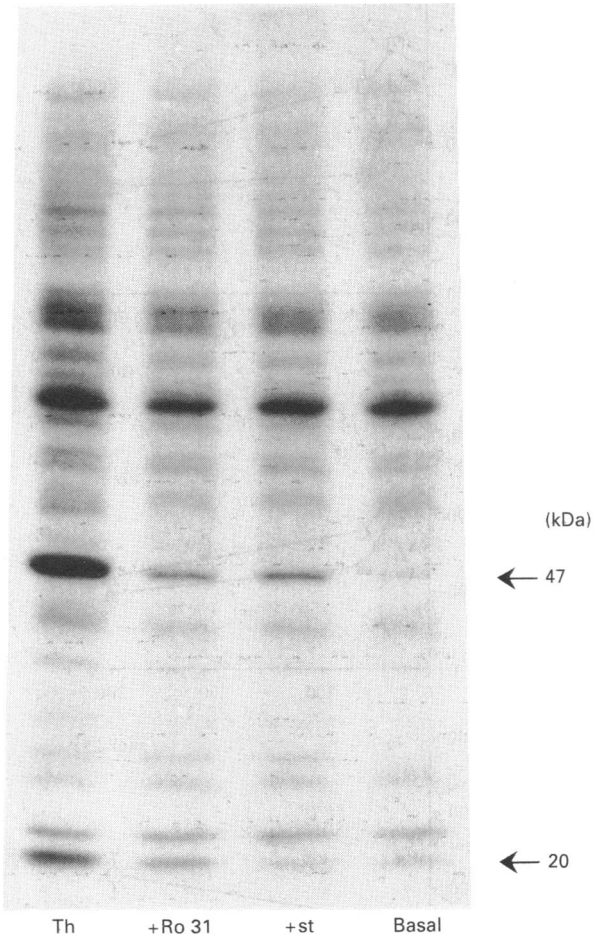

Figure 3 Comparison of inhibition of thrombin-induced phosphorylation by staurosporine and Ro 31-8220

Platelets were prepared and labelled as for Figure 1, and treated as indicated: Th, thrombin (1 unit/ml); Ro 31, Ro 31-8220 (3 μM); st, staurosporine (3 μM); the inhibitors were preincubated for 1 min before addition of thrombin. The major PKC substrate and myosin light chains are indicated by their molecular masses 47 kDa and 20 kDa respectively. This is a representative experiment of two others with essentially similar results.

with the effect of staurosporine, a non-selective kinase inhibitor (Figure 3). The autoradiograph shows that staurosporine (3 μM) inhibited by more than 90% the increase in phosphorylation of pleckstrin and myosin light chain induced by thrombin, whereas Ro 31-8220 (3 μM) inhibited pleckstrin phosphorylation to a similar extent, but only partially inhibited myosin light-chain phosphorylation. Although this concentration of staurosporine is higher than that used in our previous studies (Watson et al., 1988), we have found that the potency of staurosporine varies between batches, and maximal inhibition was observed with 3 μM in this study.

Time course of phosphorylation

It has previously been reported that phosphorylation of myosin light chain after activation of protein kinase C with phorbol ester is relatively slow (Naka et al., 1983). Stimulation of platelets with PDBu induced an increase in phosphorylation over 5 min, which was completely inhibited in the presence of Ro 31-8220 (3 μM) ($P < 0.05$) (Figure 4c). Phosphorylation of myosin light chain by thrombin (1 unit/ml) reached a peak by 30 s and decreased over 5 min, and was inhibited partially by Ro 31-8220 (3 μM) at all time points investigated, including the earliest time point of 10 s (Figure 4a). In contrast, A23187 (1 μM)-induced phosphorylation of myosin light chain reached a peak by 10 s and decreased over 5 min, and inhibition by Ro 31-8220 (3 μM) was observed after 30 s (Figure 4b), consistent with selective inhibition of a slow protein kinase C component.

Ro 31-8220 (3 μM) inhibited phosphorylation of pleckstrin induced by thrombin, A23187 and PDBu at times from 10 s to 5 min (results not shown).

Role of protein kinase C in secretion

Direct activation of protein kinase C by PDBu (300 nM) induced secretion to 3.3 ± 1.0% which was completely inhibited by Ro 31-8220. A23187 induced secretion of 5-HT from platelets in a dose-dependent manner to 50 ± 3.9% of tissue levels (Figure 5a), and this was potentiated to 70.8 ± 2.4% by PDBu. However, potentiation was most marked at 300 nM A23187, where secretion increased from 2.8 ± 1.1 to 52.5 ± 3.1% (Figure 5a). Ro 31-8220 (3 μM) inhibited secretion by A23187 (3 μM) and A23187 in the presence of PDBu to similar levels (18.5 ± 2.3 and 15.5 ± 2.7% respectively). Staurosporine (10 μM) was found to have no additional action on secretion induced by A23187 or A23187 and PDBu in the presence of Ro 31-8220 (results not shown), indicating that this component of secretion is likely to be phosphorylation-independent.

Thrombin (1 unit/ml)-induced 5-HT secretion was inhibited by Ro 31-8220 over the same concentration range at which inhibition of agonist-induced phosphorylation was observed. At a maximal concentration of Ro 31-8220, secretion induced by 1 unit of thrombin/ml was inhibited from 65.1 ± 1.3 to

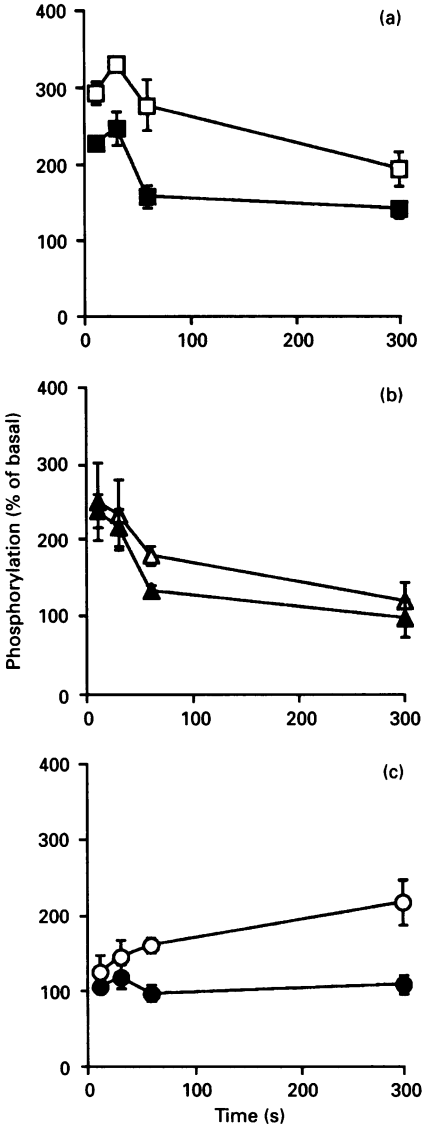

Figure 4 Effect of Ro 31-8220 on myosin light-chain phosphorylation

Platelets were prepared as for Figure 1. Platelets were stimulated with (**a**) thrombin (1 unit/ml) (□), (**b**) A23187 (3 μM) (△) or (**c**) PDBu (300 nM) (○) for various times in the presence or absence of Ro 31-8220 (3 μM), which had been preincubated for 1 min; black symbols represent the inclusion of Ro 31-8220. Electrophoresis and autoradiography were carried out as described previously. Results are expressed as means ± S.E.M. (n = 3–5).

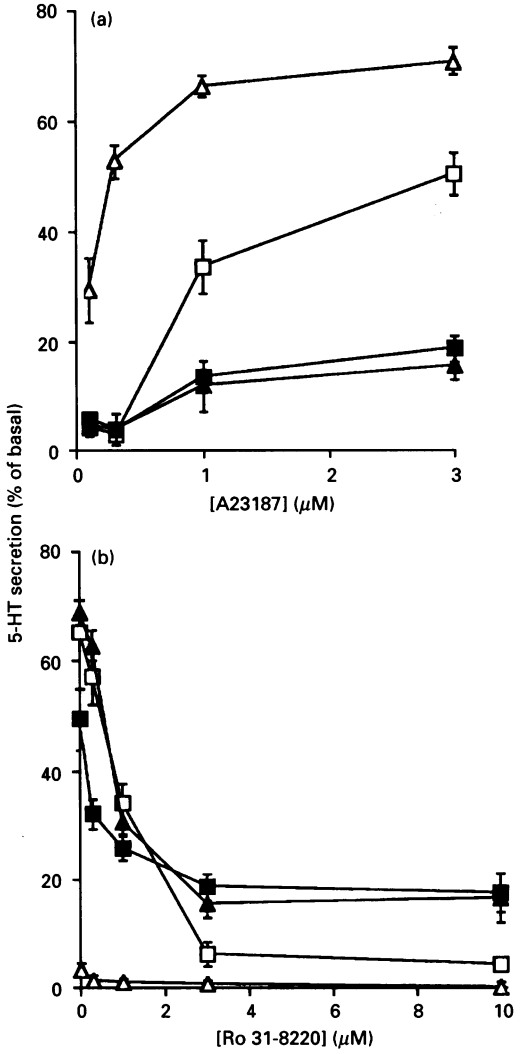

Figure 5 Effect of Ro 31-8220 on 5-HT secretion

Platelets were prepared and labelled as described in the Methods section. (**a**) Platelets were stimulated with increasing concentrations of A23187 for 1 min in the presence (△) or absence (□) of PDBu (300 nM) and in the presence or absence of Ro 31-8220 (3 μM), which had been preincubated for 1 min; black symbols represent the inclusion of Ro 31-8220. Incubation with PDBu was carried out for 1 min before addition of A23187 as appropriate. (**b**) Platelets were stimulated with thrombin (1 unit/ml) (□), A23187 (3 μM) (■), PDBu (300 nM) (△) or A23187 + PDBu (▲) in the presence of various concentrations of Ro 31-8220 as indicated; Ro 31-8220 was preincubated for 1 min before stimulation. Results are expressed as means ± S.E.M. (n = 3–6).

4.3 ± 1.2%; however, secretion was not inhibited completely ($P < 0.05$) (Figure 5b).

Role of protein kinase C in shape change and aggregation

Aggregation induced by thrombin (1 unit/ml) was slowed in the presence of 10 μM Ro 31-8220, although shape change was unaffected. Ro 31-8220 (10 μM) had no effect on concentrations of thrombin which only initiated shape change (e.g. see Figure 6). Similar responses were observed upon stimulation of platelets with A23187 (3 μM). Staurosporine (10 μM), in contrast, completely inhibited shape change and slowed aggregation induced by thrombin and A23187 to a similar extent to that seen with Ro 31-8220 (Figure 6). Aggregation induced by PDBu was relatively slow and occurred without shape change; this response was inhibited completely by Ro 31-8220 and staurosporine (Figure 6).

DISCUSSION

Selectivity of Ro 31-8220

Ro 31-8220 completely inhibited phosphorylation of pleckstrin induced by thrombin, A23187 and phorbol dibutyrate, which is mediated through protein kinase C. Staurosporine, a non-selective inhibitor of protein kinases (Watson et al., 1988), completely inhibits phosphorylation of both pleckstrin and myosin light chain induced by thrombin (Figure 3). In contrast, Ro 31-8220 only partially inhibited thrombin-induced phosphorylation of myosin light chain. Myosin light chain is a

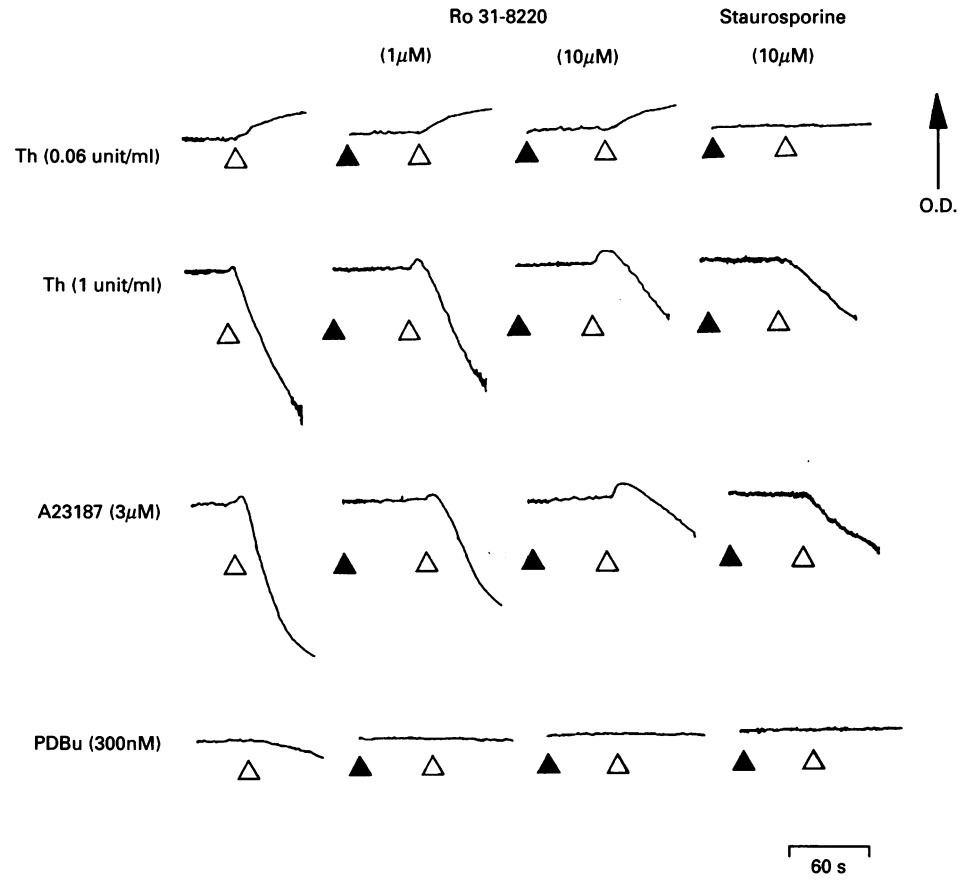

Figure 6 Effect of Ro 31-8220 on aggregation

Platelets were prepared as described in the Methods section and aggregation measurements carried out in a Born lumi-aggregometer; shape-change is indicated as an increase in optical density (O.D.) and aggregation is indicated by a decrease in O.D. Platelets were incubated for 1 min in the presence of Ro 31-8220 or staurosporine (△) before stimulation with thrombin (Th), A23187 or PDBu (▲) as indicated. This trace is representative of two experiments.

substrate for myosin light-chain kinase and protein kinase C, although these enzymes act at distinct sites (Ikebe and Hartshorne, 1985; Ikebe et al., 1986). Ro 31-8220 caused a complete inhibition of the protein kinase C component, demonstrated by the complete inhibition of phosphorylation of myosin light chain induced by PDBu at the same concentrations of Ro 31-8220 which inhibit pleckstrin phosphorylation. On the other hand, Ro 31-8220 caused only a partial inhibition of myosin light-chain phosphorylation induced by thrombin and A23187 at a maximally effective concentration of 3 μM. Siess and Lapetina (1988) have suggested in a previous study that Ca^{2+} mobilization by A23187 may prime protein kinase C under physiological conditions, as indicated by increased [^{3}H]PDBu binding after treatment of platelets with A23187 or other Ca^{2+}-mobilizing agents. This was confirmed by analysis of phosphorylation induced by the Ca^{2+} ionophore A23187: Ca^{2+} mobilization was observed to activate protein kinase C sub-maximally as shown by the increase in phosphorylation of pleckstrin (Figure 1a).

Myosin light chain is a substrate for both Ca^{2+}/calmodulin-dependent myosin light-chain kinase and for protein kinase C, although the action of each kinase occurs at distinct sites (Naka et al., 1983). Protein kinase C phosphorylates myosin light chain at threonine residues and two sites have been identified in smooth muscle; on the other hand, myosin light-chain kinase acts at a serine and a distinct threonine residue (Ikebe and Hartshorne, 1985; Ikebe et al., 1986). As myosin light-chain phosphorylation induced by thrombin and A23187 is only partially inhibited by Ro 31-8220, reported as a selective protein kinase C inhibitor (Davis et al., 1989), it would appear that myosin light chain may be phosphorylated by both myosin light-chain kinase and protein kinase C. The difference in the degree of phosphorylation induced by A23187 compared with thrombin may reflect differential activation of protein kinase C (Figure 1b). Analysis of tryptic digests have confirmed that peptide fragments phosphorylated by protein kinase C were completely inhibited by Ro 31-8220 and have shown that Ro 31-8220 appears to be selective for protein kinase C over myosin light-chain kinase (Figure 2). Thus Ro 31-8220 is more selective than staurosporine, but we cannot rule out additional effects on other kinases, some of which may be activated downstream of protein kinase C; however, we have observed that Ro 31-8220 had no effect on agonist-induced tyrosine phosphorylation (R. A. Blake, T. R. Walker and S. P. Watson, unpublished work).

Secretion

The initiation of secretion by thrombin is the result of the synergistic action of Ca^{2+} mobilization and protein kinase C activation, as a consequence of phospholipase C-mediated

phosphoinositide hydrolysis. Ro 31-8220 has been shown to be selective for protein kinase C. It can therefore be used to evaluate the interactive contribution of protein kinase C with Ca^{2+} to mediate secretion. Secretion induced by thrombin was inhibited by Ro 31-8220 to $4.3 \pm 1.2\%$, presumably revealing secretion mediated solely by Ca^{2+} mobilization (Figure 5b). A23187-induced secretion was potentiated by PDBu to a similar level to that induced by thrombin. This demonstrates that direct activation of protein kinase C synergizes with mobilized Ca^{2+}, mimicking the response to agonist-induced stimulation (Figure 5a). Ro 31-8220 (3 μM) inhibited A23187-induced secretion in the presence and absence of PDBu to similar levels, around 17%, indicating the level of secretion which is solely Ca^{2+}-mediated (Figure 5a). Secretion induced by thrombin and A23187, in the presence of Ro 31-8220 (10 μM), differs significantly (Figure 5b), which presumably reflects the ability of different spatial and temporal patterns of Ca^{2+} mobilization to induced secretion. Phosphorylation studies examining stimulation of platelets with A23187 (1 μM) have shown a sub-maximal phosphorylation of pleckstrin as a result of Ca^{2+}-induced protein kinase C activation (Figure 1a). The functional consequence of this protein kinase C activation may be that A23187-induced secretion is not simply due to Ca^{2+} mobilization, but also contains a small significant input from the protein kinase C pathway to amplify the secretory response. This is supported by the ability of Ro 31-8220 to inhibit partially the secretion induced by A23187, in the presence and absence of PDBu, at concentrations above 300 nM (Figure 5a). Direct activation of protein kinase C alone by PDBu induced a low level of secretion which was completely inhibited by Ro 31-8220 (Figure 5b). Secretion is therefore dependent on Ca^{2+} mobilization, and this is markedly potentiated by protein kinase C, the synergistic effect being the major factor in determining the extent of secretion.

Aggregation and shape change

The platelet aggregation event is composed of two components which interact synergistically, a rapid phosphorylation-independent pathway mediated by Ca^{2+} and a slower phosphorylation-dependent pathway (Watson and Hambleton, 1989). The rate of aggregation induced by thrombin was slowed by Ro 31-8220 to that obtained with staurosporine, indicating that this component is phosphorylation-independent but is potentiated by protein kinase C. In contrast, the shape-change response, which is Ca^{2+}-dependent, is unchanged (Figure 6); as staurosporine inhibits shape change, this response appears to be dependent on activation of a Ca^{2+}-mediated kinase and is independent of protein kinase C. Phosphorylation of myosin light chain has been suggested to precede shape change after stimulation of platelets with a number of agonists, suggesting that this event is required for the initial change in shape (Daniel et al., 1984). Time-course analyses of phosphorylation of pleckstrin induced by thrombin, A23187 and PDBu show that Ro 31-8220 caused inhibition at the earliest points measured (Figure 4), confirming that inhibition of protein kinase C is involved in inhibition of aggregation subsequent to shape change. Thrombin- and A23187-induced phosphorylations of myosin light chain were not significantly inhibited by Ro 31-8220 at early time points; significant inhibition occurred 30 s after addition of either agent. In contrast, phosphorylation of myosin light chain induced by PDBu increased in a linear manner over 5 min, which was significantly inhibited by Ro 31-8220 from 30 s. Phosphorylation of myosin light chain by protein kinase C would appear to occur after the initial increase induced by Ca^{2+} mobilization, as the later time points are more sensitive to inhibition by Ro 31-8220. These results suggest that protein kinase C does not play a significant role in the initial phosphorylation of myosin light chain which correlates with shape change.

It has been shown that Ro 31-8220 is a more selective inhibitor of protein kinase C than are previously reported inhibitors, allowing further investigation of the role of protein kinase C in functional responses. As protein kinase C is known to comprise a family of enzymes (Stabel and Parker, 1991), each subtype with different structures and cofactor requirements, it would be of interest to investigate the inhibitory specificity of Ro 31-8220 against each of the subtypes. Recently, three different protein kinase C subtypes have been identified in platelets by immunological techniques, namely protein kinase C-α, -β and -ζ (Crabos et al., 1991). Use of specific antibodies to these subtypes in combination with protein kinase C inhibitors would allow analysis of the functional consequences of selective inhibition of particular subtypes.

We thank Roche Products Ltd. for their kind gift of Ro 31-8220. We are grateful to Belén Rodríguez-Liñares for help with the initial studies. T.R.W. is in receipt of a B.H.F. studentship, and S.P.W. is a Royal Society University Research Fellow.

REFERENCES

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106
Crabos, M., Imber, R., Woodtli, T., Fabbro, D. and Erne, P. (1991) Biochem. Biophys. Res. Commun. **178**, 878–883
Daniel, J. L. and Adelstein, R. S. (1976) Biochemistry **15**, 2370–2377
Daniel, J. L., Molish, I. R., Rigmaiden, M. and Stewart, G. (1984) J. Biol. Chem. **259**, 9826–9831
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
Ikebe, M. and Hartshorne, D. J. (1985) J. Biol. Chem. **260**, 10027–10031
Ikebe, M., Hartshorne, D. J. and Elzinga, M. (1986) J. Biol. Chem. **261**, 36–39
Imaoka, T., Lynham, J. A. and Haslam, R. J. (1983) J. Biol. Chem. **258**, 11404–11414
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Naka, M., Nishikawa, M., Adelstein, R. S. and Hidaka, H. (1983) Nature (London) **306**, 490–492
Nishizuka, Y. (1984) Nature (London) **308**, 693–697
Nunn, D. L. and Watson, S. P. (1987) Biochem. J. **243**, 808–813
Sano, K., Takai, Y., Yamanishi, J. and Nishizuka, Y. (1983) J. Biol. Chem. **258**, 2010–2013
Siess, W. (1989) Physiol. Rev. **69**, 58–178
Siess, W. and Lapetina, E. G. (1988) Biochem. J. **255**, 309–318
Stabel, S. and Parker, P. J. (1991) Pharmacol. Ther. **51**, 71–95
Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. **135**, 397–402
Tyers, M., Rachubinski, R. A., Stewart, M. I., Varriochio, A. M., Shorr, R. G. L., Haslam, R. J. and Harley, C. B. (1988) Nature (London) **333**, 470–473
Watson, S. P. and Hambleton, S. (1989) Biochem. J. **258**, 479–485
Watson, S. P., McNally, J., Shipman, L. J. and Godfrey, P. P. (1988) Biochem. J. **249**, 345–350
Watson, S. P., Ruggerio, M., Abrahams, S. L. and Lapetina, E. G. (1986) J. Biol. Chem. **261**, 5368–5372

Received 18 May 1992/22 July 1992; accepted 30 July 1992