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Selective inhibition of protein kinase C

Effect on platelet-activating-factor-induced platelet functional responses

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The role of protein kinase C (PKC) in platelet-activating-factor (PAF)-induced platelet activation was examined by using two selective inhibitors of PKC, namely Ro 31-7549/001 and Ro 31-8220/002. Both inhibitors dose-dependently inhibited PAF-induced phosphorylation of the major 40–47 kDa protein substrate of PKC, with 50% inhibition at 4.5 μM-Ro 31-7549/001 and 0.7 μM-Ro 31-8220/002. Inhibition of PKC had no effect on maximal elevation of intracellular Ca^{2+} $[Ca^{2+}]_i$ produced by either a high or a low dose of PAF, but significantly increased the duration of the Ca^{2+} signal and the thromboxane B_2 (TxB_2) generation in high-dose PAF-stimulated platelets. The inhibitors also abrogated the effect of the PKC activator phorbol 12-myristate 13-acetate on PAF-induced $[Ca^{2+}]_i$ elevation. Sub-maximal PAF-induced dense-granule release and platelet aggregation were dose-dependently inhibited by Ro 31-7549/001 and Ro 31-8220/002. The findings suggest that endogenously activated PKC holds a bifurcating role in PAF-activated platelets, negatively affecting duration of both $[Ca^{2+}]_i$ and TxB_2 generation, and positively influencing dense-granule release and aggregation.

INTRODUCTION

Protein kinase C (PKC) is thought to hold a pivotal role in agonist-initiated signal transduction in the platelet (Nishizuka, 1984). Agonist-receptor-coupled platelet activation induces the hydrolysis of phosphoinositides, producing the two signal molecules *sn*-1,2-diacylglycerol (DAG) and Ins (1,4,5)P_3 (MacIntyre & Pollock, 1983; Agranoff *et al.* 1983). DAG is the putative endogenous activator of PKC (Lapetina *et al.*, 1985), a family of Ca^{2+}-and-phospholipid-dependent isoenzymes, which phosphorylate target proteins. In platelets their major substrate is a 40–47 kDa protein of unknown function (Tyers *et al.*, 1988). Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) activate PKC by increasing its affinity for Ca^{2+} and phospholipid (Castagna *et al.*, 1982). As a consequence, phorbol esters have been widely used to try to establish the role of PKC in signal transduction. However, owing to the use of permeabilized platelet preparations in many of these studies (Halenda *et al.*, 1985; Kaibuchi *et al.*, 1983; Mobley & Tai, 1985) and also the differences between PMA and endogenous DAG, including both their permeability and longevity (Bazzi & Nelsestuen, 1989), these studies are difficult to relate to the physiological situation. On the other hand, an inhibitor of PKC would allow the role of endogenously activated PKC to be examined.

Some of the earliest PKC inhibitors described include chlorpromazine, dibucaine and other phospholipid-interacting drugs (Mori *et al.*, 1980), including polymyxin B (Mazzei *et al.*, 1982), which inhibit PKC by competing with phospholipid. Later it was found that members of the isoquinoline sulphonamide family, particularly H-7, were capable of inhibiting PKC by competing with ATP for its binding site on PKC (Hidaka *et al.*, 1984; Yamamoto & Hidaka, 1984). In 1985 the non-steroidal triphenylacrylonitrile (TPE) tamoxifen used in the treatment of breast cancer was shown to inhibit PKC (O'Brian *et al.*, 1985), and more recently was shown to be relatively selective for PKC (Spacey *et al.*, 1990). Depending on the structure of the TPE, it inhibits PKC either at the regulatory or the catalytic domain (Bignon *et al.*, 1989). In order for the role of PKC to be unambiguous, however, an inhibitor would need to be both potent and selective for PKC above other protein kinases. Until the discovery of staurosporine (Tamaoki *et al.*, 1986) and members of the same family, including K252a (Yamada *et al.*, 1987) and UCN01 (Ruegg & Burgess, 1989), all inhibitors of PKC had low potency and, except tamoxifen, were non-selective. Staurosporine and related compounds were found to be potent inhibitors of PKC, but because they inhibited by competing with ATP for the ATP co-substrate site common to all protein kinases, they had limited selectively for PKC (Ruegg & Burgess, 1989). Since the introduction of staurosporine, other PKC inhibitors, including amino acrides (Hannun & Bell, 1988; Smal *et al.*, 1989), pseudo-substrate analogues of PKC (Ricouart *et al.*, 1989) and calphostin C (Kobayashi *et al.*, 1989), have been produced which are thought to inhibit PKC at the catalytic and regulatory sites, at the substrate-binding site and at the regulatory domain respectively.

Several potent and selective inhibitors of PKC have been described (Davis *et al.*, 1989). In this study we have used two of these, namely Ro 31-7549/001 and Ro 31-8220/002, which, although structured on staurosporine and inhibiting PKC by competing with ATP for the co-substrate site, have been demonstrated to be selective for PKC over both protein kinase A and Ca^{2+}/calmodulin dependent kinase (Davis *et al.*, 1989). We have used these two compounds in order to examine the role of endogenously activated PKC in the signal-transduction pathway in platelet-activating-factor (PAF)-stimulated rabbit platelets.

MATERIALS AND METHODS

Materials

Prostacyclin was generously provided by Dr. B. J. R. Whittle, Wellcome Laboratories, Beckenham, Kent, U.K. Stock samples were dissolved in 0.5 M-Tris/HCl (pH 10.5) at a concentration of

Abbreviations used: PKC, protein kinase C; DAG, *sn*-1,2-diacylglycerol; PMA, phorbol 12-myristate 13-acetate; TPE, triphenylacrylonitrile; PAF, platelet-activating factor; HBT, Hepes-buffered Tyrode's solution; TxB_2, thromboxane B_2; $[Ca^{2+}]_i$, intracellular Ca^{2+}; 5-HT, 5-hydroxytryptamine; IC_{50}, concn. giving 50% inhibition; PLC, phospholipase C.

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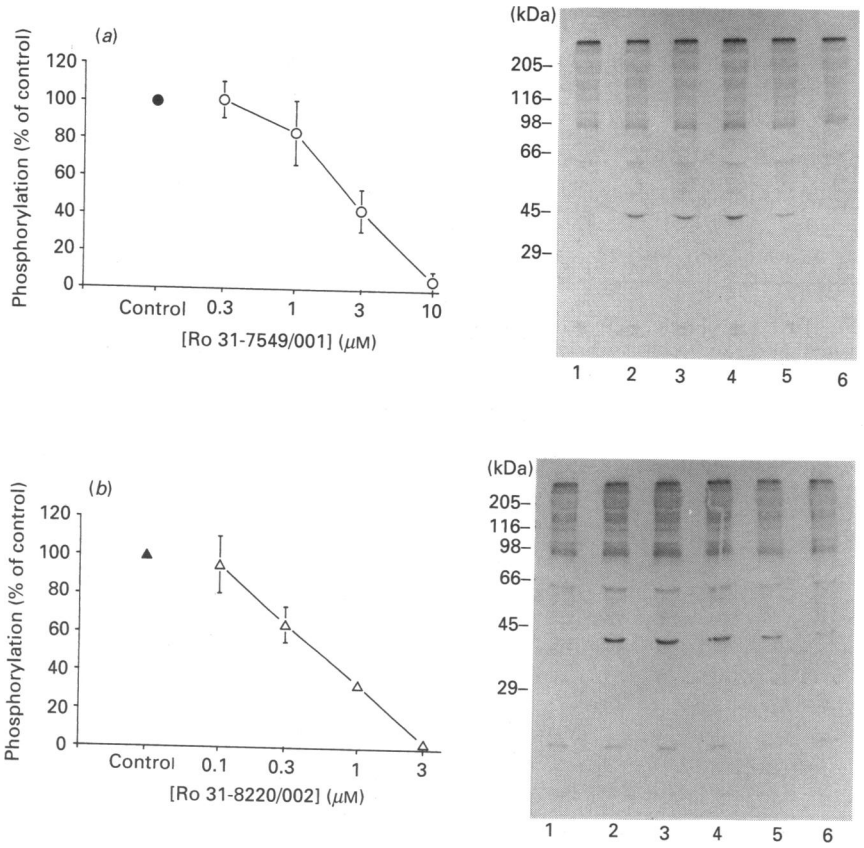

Fig. 1. Inhibition of PAF-induced phosphorylation of the 40–47 kDa substrate of PKC by Ro 31-7549/001 (*a*) and Ro 31-8220/002 (*b*)

The right panels in (*a*) and (*b*) are autoradiographs representing: in track 1, phosphorylation in resting platelets; in track 2, 300 nM-PAF-induced platelet phosphorylation in the presence of the respective PKC inhibitor vehicle; and in tracks 3–6 respectively, either a 20 min preincubation with 0.3 μM-, 1 μM-, 3 μM-, 10 μM-Ro 31-7549/001 (*a*) or 0.1 μM-, 0.3 μM-, 1 μM-, 3 μM-Ro 31-8220/002 (*b*), followed by 300 nM-PAF stimulation for 30 s. On the left, phosphorylation was quantified by liquid-scintillation counting of the excised [^{32}P]P_i-labelled PKC substrate. The data are expressed as phosphorylation of the PKC substrate, as a percentage of that phosphorylated by 300 nM-PAF alone (●; control), and each point is the mean ± S.E.M. of three separate experiments.

40 μg/40 μl. Working samples were diluted in 0.01 M-Tris/HCl (pH 10.5) and kept on ice. 5-Hydroxy[^{14}C]tryptamine (5-H[^{14}C]T) creatinine sulphate (54 mCi/mmol; 50 μCi/ml) and [^{32}P]P_i (5 mCi/ml) were purchased from Amersham International. X-ray film was purchased from Fuji. Fura-2 AM (acetoxymethyl ester; cell-permeant) was obtained from Molecular Probes, Eugene, OR, U.S.A., and a stock solution at a concentration of 5 mM in anhydrous dimethyl sulphoxide was stored at −20 °C. Thromboxane B_2 (TxB_2) standard was obtained from Sigma, [^{3}H]TxB_2 (125 Ci/mmol; 50 nCi/ml) from New England Nuclear and anti-(rabbit TxB_2) antibody was generously supplied by Dr. F. Carey, ICI, Macclesfield, U.K. PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was supplied by Bachem (Bubendorf, Switzerland). PMA was obtained from Sigma, dissolved in dry acetone and stored at −20 °C. PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002, were kindly given by Dr. J. S. Nixon, Roche, Welwyn, Herts., U.K. Both PKC inhibitors were dissolved at 10 mM in dimethyl sulphoxide, and further dilutions were made in Hepes-buffered Tyrode's solution (HBT).

Methods

Preparation of platelets. Washed rabbit platelets were prepared as described for human platelets by Poll & Westwick (1986*a*) by a modification of an original method by Blackwell *et al.* (1982). The resulting platelet pellet from this preparation was suspended in HBT (10 mM-Hepes, 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 0.5 mM-Na_2HPO_4, 5.5 mM-glucose and 0.25% BSA, pH 7.4) and the platelet count adjusted. All experiments were carried out at 37 °C in the presence of 1 mM extracellular Ca^{2+}.

Protein phosphorylation. Platelets (8×10^9/ml) were incubated for 2 h with 200 μCi of [^{32}P]P_i/ml of platelets, washed in HBT, and the platelet count was adjusted to 10^9/ml. Samples of platelets (300 μl; 10^9 cells/ml) were preincubated with a dose range of the PKC inhibitors or their vehicle for 20 min before activation with 300 nM-PAF. Activation was terminated with 300 μl of 2 × Laemmli (1970) SDS reducing gel sample buffer (100 °C) 30 s after PAF addition. The samples were then boiled for 5 min, and centrifuged at 8000 *g* for 2 min to remove any particles. Proteins (100 μg/track) were separated by SDS/PAGE (10% gel), and protein bands and molecular markers were stained with Coomassie Blue. The gels were then dried and [^{32}P]phosphorylated bands were detected by autoradiography. The phosphorylated bands were excised and quantified by liquid-scintillation counting.

Determination of intracellular Ca^{2+} concn. ($[Ca^{2+}]_i$). Platelets were loaded with 2.5 μM fura-2 AM for 30 min at 37 °C, the platelet suspension was then washed in HBT to remove excess fura-2 AM, and the platelet count adjusted to 2×10^8/ml. In order to measure the changes in $[Ca^{2+}]_i$, fluorescence readings were recorded with an Aminco–Bowman spectrophotofluorimeter (excitation at 339 nm, emission at 500 nm; 4 nm slit width) fitted with a thermostatically controlled cuvette compartment holder with stirring attachment. The Ca^{2+}/fura-2-fluorescence was calibrated as previously described (Grynkiewicz

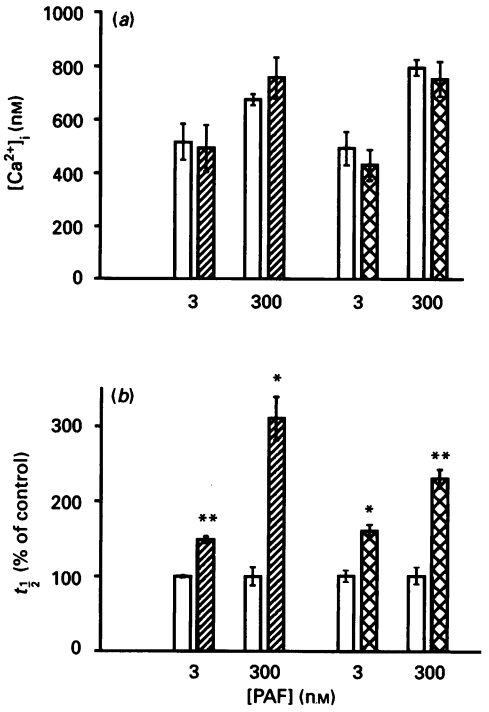

Fig. 2. Effect of the PKC inhibitors on maximal $[Ca^{2+}]_i$ elevation (*a*) and the duration of $[Ca^{2+}]_i$ elevation (*b*) in 3 nM- and 300 nM-PAF-stimulated platelets

Platelets were incubated with 10 μM-Ro 31-7549/001 ▨, 3 μM-Ro 31-8220/002 (⊠) or their appropriate vehicle (□, control; < 0.1% dimethyl sulphoxide) 20 min before addition of PAF. $[Ca^{2+}]_i$ is the maximal elevation of cytosolic Ca^{2+} after subtracting the basal pre-PAF value. $t_{\frac{1}{2}}$ is the time for Ca^{2+} to decrease to half-maximal above basal levels; results are expressed as percentages of the control $t_{\frac{1}{2}}$.

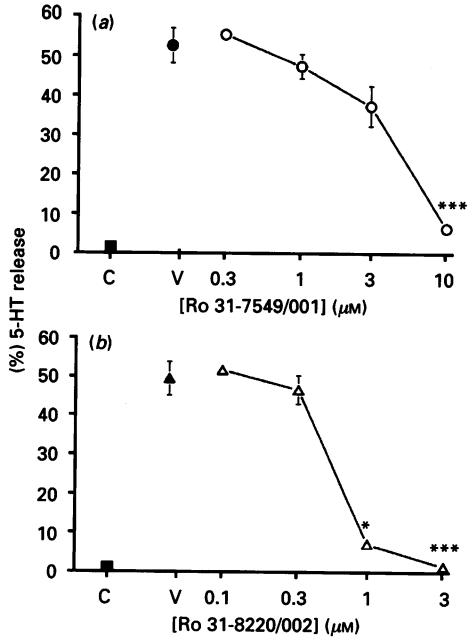

Fig. 3. Effect of the PKC inhibitors on dense-granule release induced by PAF

Platelets were treated for 20 min with Ro 31-7549/001 (○) or Ro 31-8220/002 (△) before activation with 3 nM-PAF. The percentage 5-HT release induced by the highest concentration of each PKC inhibitor alone (C) is shown as ■ in each panel, and the effect of the PKC-inhibitor vehicle (V) on 3 nM-PAF-induced granule release is shown as ● and ▲ for Ro 31-7549/001 and Ro 31-8220/002 respectively. Dense-granule release was terminated 3 min after addition of PAF.

et al., 1985; Pollock *et al.*, 1986). Addition of the PKC inhibitors or their vehicle was made 20 min before stimulation with PAF.

Measurement of TxB_2 generation. This was done with 1 ml samples of platelets (2×10^8/ml), and PKC inhibitor/vehicle was added 20 min before PAF. To determine TxB_2 generation, subsamples of platelets (400 μl) were withdrawn at 3 min into 40 μl of ice-cold 100 mM-EDTA and 30 μM-indomethacin. The samples were centrifuged for 2 min at 12000 *g*, the supernatant was removed and the TxB_2 content was determined by radioimmunoassay (Johnston *et al.*, 1984).

Measurement of 5-H[^{14}C]T release. Platelets (8×10^8/ml) were incubated with 200 nCi of 5-H[^{14}C]T/ml, then washed in HBT, and the platelet count was adjusted to 2×10^8/ml. Addition of a PKC inhibitor or its vehicle control was made 20 min before stimulation with 3 nM-PAF. The 5-HT release from the platelets was terminated as described for termination of TxB_2 generation. After the samples were centrifuged two 100 μl samples of the supernatant were removed and the amount of 5-H[^{14}C]T was determined by liquid-scintillation spectroscopy. Percentage 5-HT release was determined as previously described (Holmsen & Dangelmaier, 1989).

Platelet aggregation. This was continually monitored by a twin-channel Ca^{2+} aggregometer, and the results were expressed as a percentage increase in light transmission. Samples of platelets (2×10^8/ml) were pretreated with the PKC inhibitors for 20 min before addition of PAF. The 100% light transmission was that produced by diluting the platelets to 2×10^7/ml. Platelets were continually stirred throughout experimentation at 900 rev./min.

Statistics. Data are means ± S.E.M. of at least three experiments each performed in triplicate. To determine the levels of significance between sets of data, a paired Student's *t* test was employed. The levels of significance are denoted by $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. The Bonferroni correction was applied where necessary (Wallenstein *et al.*, 1980).

RESULTS

Inhibition of phosphorylation of the major substrate of PKC

A 20 min preincubation with the selective PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002 inhibited 300 nM-PAF-induced phosphorylation of the 40–47 kDa major PKC substrate in a dose-dependent manner (Figs. 1*a* and 1*b*). Ro 31-7549/001 inhibited phosphorylation with an IC_{50} (concn. giving 50% inhibition) of 2.5 μM, and Ro 31-8220/002 inhibited phosphorylation with an IC_{50} of 0.45 μM.

Effect of PKC inhibition on PAF-induced functional responses

$[Ca^{2+}]_i$ elevation. The effect of inhibition of PKC on $[Ca^{2+}]_i$ elevation was investigated by using platelets stimulated with both sub-maximal (3 nM) and maximal (300 nM) doses of PAF (Fig. 2). Doses of 10 μM-Ro 31-7549/001 and 3 μM-Ro 31-8220/002 were used, as these concentrations were found to inhibit PKC activation by > 90% (Fig. 1). Preincubation with either Ro 31-7549/001 or Ro 31-8220/002 for 20 min had no effect on maximal $[Ca^{2+}]_i$ elevation induced by either 3 nM- or 300 nM-PAF; however, both inhibitors significantly increased the duration of the Ca^{2+} signal (Fig. 2). Moreover, whereas in 300 nM-PAF-stimulated platelets inhibition of PKC caused up to a 180% increase in duration of the Ca^{2+} signal, it only caused about a 50% increase with 3 nM-PAF (Fig. 2*b*).

Reversal of PMA-induced PKC activation by using Ro 31-7549/001 and Ro 31-8220/002. The ability of the PKC inhibitors

Table 1. Abrogation of PMA-induced inhibition of $[Ca^{2+}]_i$ elevation by Ro 31-7549/001 and Ro 31-8220/002

Platelets were preincubated with the PKC inhibitors or vehicle 20 min before addition of the PKC activator PMA (30 nM) and then incubated for 2 min before stimulation with 3 nM-PAF. $[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the maximal post-PAF value. Data were taken from three separate experiments each performed in triplicate.

PKC inhibitor (μM)	PMA (nM)	PAF (nM)	$[Ca^{2+}]_i$ (nM)
–	–	3	432±33
–	30	3	12±5
Ro 31-7549/001			
0.3	30	3	24±10
1	30	3	93±41
3	30	3	562±22
10	30	3	524±53
Ro 31-8220/002			
0.1	30	3	5±4
0.3	30	3	42±8
1	30	3	450±86
3	30	3	441±47

to abrogate the effect of PKC activation was studied by investigating their ability to reverse the effect of the PKC activator PMA on PAF-induced $[Ca^{2+}]_i$ elevation (Table 1). Activation of PKC with 30 nM-PMA inhibited $[Ca^{2+}]_i$ elevation in PAF-stimulated platelets. A 20 min preincubation with the PKC inhibitors completely abrogated the effect on $[Ca^{2+}]_i$ caused by PMA.

TxB_2 generation. With 10 μM-Ro 31-7549/001 and 3 μM-Ro 31-8220/002 (concentrations which inhibited PKC substrate phosphorylation by > 90%), the effect of inhibition of PKC on

Table 2. Effect of 10 μM-Ro 31-7549/001 and 3 μM-Ro 8220/002 on TxB_2 generation in 30 nM-PAF-stimulated platelets and the abrogation of PMA-induced inhibition of TxB_2 generation by the PKC inhibitors

Platelets were preincubated with the PKC inhibitors or vehicle 20 min before addition of the PKC activator PMA (30 nM) which was incubated for 2 min before stimulation with 30 nM-PAF. TxB_2 generation was measured at 3 min after PAF.

	TxB_2 (pmol/10^8 platelets)	
	Vehicle	+30 nM-PMA
Control	15.6±1.8	4.4±0.5**
10 μM-Ro 31-7549/001	65.1±10**	51.3±1.9**
3 μM-Ro 31-8220/002	50.9±9.9*	56.2±10*

TxB_2 generation was investigated in platelets stimulated with 30 nM-PAF (Table 2). Both PKC inhibitors caused a 3–5-fold increase in TxB_2 induced by 30 nM-PAF. Similarly to the results for $[Ca^{2+}]_i$ elevation, inhibition of TxB_2 generation by PMA was abrogated by the PKC inhibitors (Table 2). These findings indicate that the inhibition of $[Ca^{2+}]_i$ elevation and TxB_2 generation by PMA in PAF-stimulated platelets is mediated by PKC and can be reversed by preincubation with either PKC inhibitor.

5-HT release. The 5-HT release induced by 3 nM-PAF was dose-dependently inhibited when platelets were subjected to a 20 min preincubation with increasing concentrations of Ro 31-7549/001 (0.3, 1, 3, 10 μM) or Ro 31-8220/002 (0.1, 0.3, 1, 3 μM). The IC_{50} for Ro 31-7549/001 was 4.7 μM, and for Ro 31-8220/002 it was 0.55 μM (Fig. 3).

Aggregation. Aggregation of platelets induced by a submaximal concentration of PAF (1 nM) was dose-dependently inhibited with increasing concentrations of either PKC inhibitor, with almost total inhibition of platelet aggregation at 10 μM-Ro

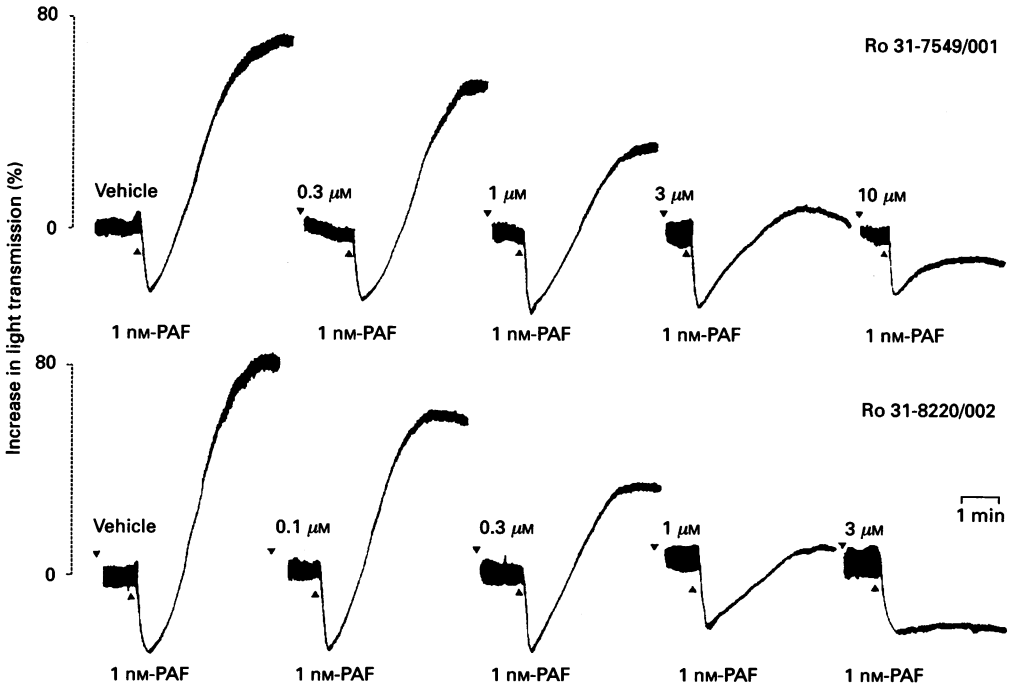

Fig. 4. Traces showing the effect of PKC inhibition on PAF-induced platelet shape change and aggregation

Platelets were preincubated for 20 min with a dose range of Ro 31-7549/001 (0.3–10 μM, top panel) or Ro 31-8220/002 (0.1–3 μM, lower panel). The second arrow in each trace indicates addition of PAF.

31-7549/001 or 3 μM-Ro 31-8220/002. The IC_{50} for Ro 31-7549/001 was 1.8 μM and for Ro 31-8220/002 it was 0.47 μM (Fig. 4). However, the PKC inhibitors did not affect 1 nM-PAF-induced shape change in platelets.

DISCUSSION

The use of the selective PKC inhibitors Ro 31-7549/001 and Ro 31-8220/002 (Davis *et al.*, 1989) in this study has demonstrated that PKC has a bifurcating role in PAF-induced platelet activation. Earlier studies by both ourselves (Murphy *et al.*, 1990, 1991) and others (Conolly *et al.*, 1990; Watson & Lapetina, 1985) demonstrated that activating PKC with exogenous activators such as phorbol esters or membrane-permeant DAG, followed by receptor-operated platelet activation, leads to an inhibition of the two products of phospholipase C (PLC) activity, namely Ins(1,4,5)P_3 and DAG. This suggests that activated PKC is exerting a negative-feedback role which results in the inhibition of PLC either by inactivation of the receptor by phosphorylation, or at the level of the GTP-binding proteins coupling PLC to the receptor, or as a direct inactivation of the PLC enzyme.

In addition, other forms of platelet activation are inhibited as a consequence of exogenous PKC activation in agonist-stimulated platelets including $[Ca^{2+}]_i$ elevation (Poll & Westwick, 1986*a*; Valone & Johnson, 1987; Murphy & Westwick, 1989) and TxB_2 generation (Murphy & Westwick, 1989, 1990; Wheeler-Jones *et al.*, 1990). Inhibition of both these responses is possibly a direct result of inhibition of PLC and the production of the two signal molecules Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3 causes mobilization of Ca^{2+} from intracellular stores (Brass & Joseph, 1985; Berridge & Irvine, 1984), and together with Ins(1,3,4,5)P_4 possibly regulates second-messenger-operated Ca^{2+} channels (Irvine, 1989, 1990). The inhibition of receptor-operated Ca^{2+} influx and Ca^{2+} mobilization would prevent the activation of Ca^{2+}-dependent phospholipase A_2 (PLA_2), which is probably responsible for most of the TxB_2 production (Mahaevappa & Holub, 1986). In addition, inhibition of PLC would remove the second source of arachidonic acid production and therefore TxB_2 generation, which is the action of DAG lipase on DAG (Bell *et al.*, 1979; Mahaevappa & Holub, 1986). However, recently there is increasing evidence to suggest that PLA_2 is also coupled to receptors by intermediate G-proteins, and it may be that PKC is exerting a negative feedback on PLA_2 via these G-proteins or indeed acting directly on PLA_2 (Silk *et al.*, 1990; Kajiyama *et al.*, 1989).

Nevertheless, if endogenously activated PKC is also exerting this negative-feedback effect, then inhibition of PKC would be expected to potentiate both $[Ca^{2+}]_i$ elevation and TxB_2 generation in receptor-activated platelets. In this study, two selective inhibitors of PKC employed at a concentration which caused greater than 90% inhibition of PKC substrate phosphorylation considerably potentiated the duration of $[Ca^{2+}]_i$ elevation, particularly at the higher concentrations of PAF; however, the maximal $[Ca^{2+}]_i$ elevation was not significantly altered. These results suggest that a potentiation of maximal $[Ca^{2+}]_i$ elevation with either PKC inhibitor is not seen in high-dose PAF-stimulated platelets, either because the fluorescent dye used in this study has become saturated, or because maximal elevation of $[Ca^{2+}]_i$ has been achieved and further elevation of Ins(1,4,5)P_3 cannot release any additional Ca^{2+}, but simply increases the duration of the signal molecule. Another possibility is that the Ins(1,4,5)P_3 5-phosphatase is normally activated by PKC, thus rapidly decreasing the cytosolic concentration of Ins(1,4,5)P_3. We have recently demonstrated that staurosporine, the non-selective PKC inhibitor, produces a significant enhancement of the Ca^{2+} signal (Murphy *et al.*, 1991), which was accompanied by a 2–3-fold increase in the products of PLC activation, namely Ins(1,4,5)P_3 and DAG.

The increase in duration of $[Ca^{2+}]_i$ elevation relates well to earlier findings that activation of PKC decreases the duration of $[Ca^{2+}]_i$ elevation either by increasing $[Ca^{2+}]_i$ efflux or decreasing $[Ca^{2+}]_i$ influx or mobilization (Poll & Westwick, 1986*b*; Pollock *et al.*, 1987), therefore removal of the effect of PKC activation with an inhibitor has produced the opposite effect. The more pronounced effect at the higher concentration of PAF may be due to inhibition of a greater negative feedback induced by a higher concentration of PAF; indeed, without this negative feedback at the higher concentration of PAF it is possible that Ca^{2+} may reach cytotoxic concentrations. As predicted, if endogenously activated PKC is exerting a negative feedback over PLC and/or PLA_2, TxB_2 generation was potentiated by inhibition of PKC. Moreover, *in vivo* the 3–5-fold increase in TxB_2 production, and thus thromboxane A_2 and prostaglandin endoperoxides, could have considerable effects, depending on the availability of prostacyclin synthase or vascular tissue responsive to the vasoconstrictor actions of thromboxane A_2 (Moncada & Higgs, 1986).

In contrast with the above results, this study has demonstrated that two selective inhibitors of PKC are potent inhibitors of dense-granule release and platelet aggregation, with similar IC_{50} values to those for the inhibition of PAF-induced phosphorylation of 40–47 kDa protein in intact platelets. These findings, together with the data demonstrating the specificity of these compounds, support the contention that inhibition of platelet dense-granule release and aggregation is a result of an inhibition of platelet PKC. In support of this, previous studies have shown that activation of PKC with a phorbol ester alone causes both dense-granule release and platelet aggregation (Zucker *et al.*, 1974; Castagna *et al.*, 1985), and it has been suggested that there is a direct link between phosphorylation of the major 40–47 kDa substrate of PKC and dense-granule release and aggregation (Rink *et al.*, 1983; Castagna *et al.*, 1985; Watson *et al.*, 1987).

Our results suggest that the relationship between PKC and $[Ca^{2+}]_i$ or TxB_2 generation is different from that between PKC and dense-granule release or platelet aggregation. In addition, the results indicate that the regulation of dense-granule release and aggregation by PKC is possibly independent of $[Ca^{2+}]_i$ or TxB_2 generation. Indeed, there appears to be a more direct relationship between PKC-induced protein phosphorylation and either dense-granule release or aggregation.

Comparing the effects of these selective PKC inhibitors on PAF-induced platelet activation with the effects of the non-selective PKC inhibitor staurosporine, many of the findings were similar. Both selective and non-selective inhibitors potentiated TxB_2 generation and the duration of the Ca^{2+} signal (Murphy *et al.*, 1991; Watson *et al.*, 1987), while inhibiting 5-HT release (Murphy & Westwick, 1989) and platelet aggregation (Oka *et al.*, 1987; Watson *et al.*, 1987; Schachtele *et al.*, 1988). However, whereas in platelets activated with sub-maximal concentrations of PAF staurosporine was found to inhibit platelet aggregation only partially (Watson & Hambleton, 1989), Ro 31-7549/001 and Ro 31-8220/002 caused total inhibition of aggregation while not affecting platelet shape change. It is therefore possible that staurosporine was inhibiting another protein kinase which was counteracting the effect of PKC inhibition on platelet aggregation. For example, increasing cyclic AMP levels activates protein kinase A, which inhibits platelet aggregation (Blache *et al.*, 1987); inhibition of protein kinase A by staurosporine would therefore remove this inhibitory effect and may therefore lead to only a partial possible inhibition of aggregation by staurosporine. The findings suggest therefore that sub-maximal-dose PAF-

induced platelet aggregation is PKC-dependent, whereas PAF-induced platelet shape change is PKC-independent.

In conclusion, the use of selective PKC inhibitors in PAF-stimulated rabbit platelets has demonstrated a bifurcating role for PKC. Ro 31-7549/001 and Ro 31-8220/002 were both selective inhibitors of platelet PKC and were potent inhibitors of PAF-induced dense-granule release and platelet aggregation, while producing a significant prolongation of the Ca^{2+} signal and a considerably enhanced TxB_2 generation.

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