Thromboxane-induced phosphatidate formation in human platelets

Relationship to receptor occupancy and to changes in cytosolic free calcium

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1. The inter-relationships between receptor occupancy, inositol phospholipid metabolism and elevation of cytosolic free Ca^{2+} in thromboxane A_2 -induced human platelet activation were investigated by using the stable thromboxane A_2 mimetic, 9,11-epoxymethanoprostaglandin H_2 , and the thromboxane A_2 receptor antagonist, EPO45. 2. 9,11-Epoxymethanoprostaglandin H_2 stimulated platelet phosphatidyl-inositol metabolism as indicated by the rapid accumulation of [^{32}P]phosphatidate and later accumulation of [^{32}P]phosphatidylinositol in platelets pre-labelled with [^{32}P]P_i. These effects of 9,11-epoxymethanoprostaglandin H_2 were concentration-dependent and half-maximal [^{32}P]phosphatidate formation occurred at an agonist concentration of $54 \pm 8 \text{ nM}$. 3. With platelets labelled with the fluorescent Ca^{2+} indicator quin 2, resting cytosolic free Ca^{2+} was $86 \pm 12 \text{ nM}$. 9,11-Epoxymethanoprostaglandin H_2 induced a rapid, concentration-dependent elevation of cytosolic free Ca^{2+} to a maximum of $300\text{--}700 \text{ nM}$. Half-maximal stimulation was observed at an agonist concentration of $80 \pm 23 \text{ nM}$. 4. The thromboxane A_2 receptor antagonist EPO45 selectively inhibited 9,11-epoxymethanoprostaglandin H_2 -induced [^{32}P]phosphatidate formation and elevation of cytosolic free Ca^{2+} , indicating that both events are sequelae of receptor occupancy. 5. Human platelets contain a single class of stereospecific, saturable, high affinity ($K_D = 70 \pm 13 \text{ nM}$) binding sites for 9,11-epoxymethano[^3H]prostaglandin H_2 . 6. The concentration–response curve for receptor occupancy (9,11-epoxymethano[^3H]prostaglandin H_2 binding) is similar to that for 9,11-epoxymethanoprostaglandin H_2 -induced [^{32}P]phosphatidate formation and for elevation of cytosolic free Ca^{2+} . 7. These observations indicate that human platelet thromboxane A_2 receptor occupation is closely linked to inositol phospholipid metabolism and to elevation of cytosolic free Ca^{2+} . Both such events may be necessary for thromboxane A_2 -induced human platelet activation.

The arachidonic acid metabolites prostaglandin G_2 , prostaglandin H_2 and thromboxane A_2 are potent inducers of human platelet aggregation and secretion (Hamberg *et al.*, 1974, 1975). All three compounds appear to activate platelets by combining with the same plasma membrane located receptor, termed the thromboxane A_2 receptor as

Abbreviations used: 9,11-epoxymethanoPGH₂, 9 α ,11 α -epoxymethanoprostaglandin H_2 ; Ins, inositol; PtdIns, phosphatidylinositol.

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thromboxane A_2 is the most potent endogenous agonist (MacIntyre, 1981; Armstrong *et al.*, 1983). In view of the patho-physiological importance of arachidonate metabolites in haemostasis and occlusive vascular diseases (Smith, 1980) the mechanisms whereby those compounds induce platelet activation are of considerable interest. Mechanistic studies utilizing prostaglandin G_2 or prostaglandin H_2 are complicated by the rapid conversion, either enzymically or non-enzymically, of these compounds to an array of products, many of which possess biological activity (Christ-Hazelhof

et al., 1976; Moncada & Vane, 1979). Similarly, mechanistic studies using thromboxane A_2 are hindered by its instability ($t_{1/2} = 30$ s at 37°C and pH 7.4) and rapid hydrolysis to the relatively inert metabolite thromboxane B_2 (Hamberg *et al.*, 1975). These difficulties can be overcome by the use of stable compounds such as 9,11-epoxy-methanoPGH₂ (U44069), a stable prostaglandin endoperoxide analogue (Bundy, 1975) which binds to the platelet thromboxane A_2 receptor and thus induces platelet activation by acting as a thromboxane A_2 -mimetic (MacIntyre, 1981; Armstrong *et al.*, 1983).

In platelets, the major biochemical intermediaries that translate agonist-receptor combination into the final cellular response are believed to be cyclic AMP and cytosolic free Ca^{2+} . An elevated intracellular cyclic AMP concentration is certainly associated with inhibition of platelet function (Salzman & Neri, 1969) and there is much controversy as to whether a reduction in the basal intracellular cyclic AMP concentration can lead to platelet activation (Haslam *et al.*, 1978). That platelet activation is associated with, and perhaps mediated by, an elevation of cytosolic free Ca^{2+} was indicated by studies using artificial means of increasing cytosolic free Ca^{2+} (Feinstein & Fraser, 1975; Knight & Scrutton, 1980). This theory has been confirmed by direct estimation of cytosolic free Ca^{2+} using the membrane-permeant calcium indicator dye quin 2 (Rink *et al.*, 1981). Such an elevation of cytosolic free Ca^{2+} may arise by mobilization of Ca^{2+} from intracellular stores or by influx of extracellular Ca^{2+} , presumably through some form of calcium channel (Gerrard *et al.*, 1981; Owen & Le Breton, 1981).

Michell (1975) proposed that the biochemical basis underlying Ca^{2+} flux is agonist-induced turnover of membrane PtdIns. There is evidence that ADP, collagen and thrombin stimulate PtdIns turnover in platelets (Lloyd *et al.*, 1972; Lloyd & Mustard, 1974; Rittenhouse, 1982) and the mechanisms of the thrombin effect have been analysed in detail, although rarely using human platelets (Lapetina, 1982). Agonist-receptor combination can activate a PtdIns-specific phospholipase C which hydrolyses PtdIns to diacylglycerol and Ins-1- P . Diacylglycerol may then be phosphorylated by diacylglycerol kinase to yield phosphatidate, which in turn can be reconverted to PtdIns to complete the metabolic cycle, or can be acted upon by a phosphatidate-specific phospholipase A_2 to yield lysophosphatidate and arachidonic acid (Billah *et al.*, 1981). Neufeld & Majerus (1983), however, contend that conversion of phosphatidate to lysophosphatidate does not readily occur in human platelets. Both phosphatidate and lysophosphatidate possess calcium ionophoric activity and may be

the 'effector' molecules that actually translocate Ca^{2+} from intracellular or extracellular sources (Tyson *et al.*, 1976; Gerrard *et al.*, 1979). More recent evidence indicates that breakdown of polyphosphoinositides, PtdIns(4,5) P_2 and PtdIns(4) P , rather than of PtdIns may be the initial event that follows receptor occupancy (Agranoff *et al.*, 1983; Berridge, 1983; Drummond *et al.*, 1984). These polyphosphoinositides are known to bind Ca^{2+} avidly (Dawson & Hauser, 1970) and can be degraded by phospholipase C type enzymes to form diacylglycerol and Ins-1,4- P_2 or Ins-1,4,5- P_3 (Irvine, 1982). Theoretically, receptor-mediated degradation of phosphoinositides by phospholipase C could participate in Ca^{2+} flux in several ways: (1) by directly liberating sufficient Ca^{2+} to mediate the cellular response; (2) by generating Ins-1,4,5- P_3 which may act as a second messenger to mobilize internal Ca^{2+} (Streb *et al.*, 1983); (3) by generating sufficient diacylglycerol and subsequently phosphatidate to translocate Ca^{2+} ; (4) by directly liberating sufficient Ca^{2+} to activate PtdIns-specific phospholipase C which, in turn, causes degradation of PtdIns and subsequent phosphatidate accumulation. Besides its role in platelet Ca^{2+} flux, stimulated inositol phospholipid metabolism may be involved in platelet activation by virtue of forming diacylglycerol. Platelets contain a calcium-dependent, diacylglycerol-activated protein kinase, termed protein kinase C (Kawahara *et al.*, 1980). This enzyme governs the phosphorylation of specific proteins, although these appear to be implicated mainly in platelet degranulation (Kaibuchi *et al.*, 1982) rather than in the initial stages of platelet activation.

In the present study, using a stable thromboxane A_2 -mimetic and a thromboxane A_2 antagonist, we investigated the relationship between thromboxane A_2 receptor occupation, stimulated inositol phospholipid metabolism and stimulated Ca^{2+} flux in order to gain some insight into the molecular mechanisms that link thromboxane receptor occupancy to platelet activation.

Experimental

Preparation of platelet-rich plasma

Blood was obtained from the antecubital vein of healthy human volunteers, predominantly those who denied having taken drugs known to affect platelet function for at least 7 days. In some specific instances blood was obtained from donors who had taken aspirin (600 mg by mouth) 2 days previously. Blood was collected into plastic tubes containing 10% (v/v) trisodium citrate (0.13 M) and centrifuged (1000g, 4 min, 15–20°C). The supernatant platelet-rich plasma was aspirated into plastic tubes.

Radioligand binding studies

All procedures were carried out essentially as described by Armstrong *et al.* (1983). Briefly, platelet-rich plasma was centrifuged (1600g, 10 min, 15–20°C) in the presence of prostaglandin E_1 (1.7×10^{-8} M) and indomethacin (10^{-5} M), and the resulting pellet was resuspended in a calcium-free, phosphate-free citrate buffer, pH 7.5, at 5×10^8 platelets/ml. Platelet suspension (1 ml) was then added to Eppendorf tubes containing various concentrations of 9,11-epoxymethano[^3H]PGH $_2$ in the presence or absence of excess unlabelled 9,11-epoxymethanoPGH $_2$ and incubated at room temperature for 4 min. Incubations were terminated by centrifugation (15000g, 2 min) and rapid removal of the cell-free supernatant with a Pasteur pipette. Pellets were digested by incubation with hyamine hydroxide (1 M, 50°C, 5 min) and neutralized by addition of HCl prior to transferring to scintillation vials.

Phospholipid studies

Those studies were carried out essentially as previously described (MacIntyre & Pollock, 1983). Briefly, platelet-rich plasma was centrifuged (800g, 10 min, 15–20°C) in the presence of prostaglandin I_2 (3×10^{-7} M) and the resultant pellet resuspended in calcium-free, phosphate-free, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4, and incubated (37°C, 90 min) with carrier-free [^{32}P]orthophosphate (30 $\mu\text{Ci/ml}$). Platelets were then pelleted by centrifugation (800g, 10 min, 15–20°C) in the presence of prostaglandin I_2 (3×10^{-7} M). Pellets were resuspended in 1.5-fold the labelling volume of fresh buffer and aliquots (0.4 ml, containing 0.5–1 mg of protein) were dispensed into plastic tubes at 37°C. Reactions were initiated by the addition of various concentrations of 9,11-epoxymethanoPGH $_2$ (4 μl) or vehicle (4 μl) in control samples. Antagonists or appropriate vehicle (<10 μl) were incubated with platelets for 60 s before the addition of 9,11-epoxymethanoPGH $_2$. Reactions were terminated by transferring the entire platelet sample into a glass test tube containing 2 ml of chloroform/methanol/10 M-HCl (25:50:4, by vol.) at room temperature (Lloyd *et al.*, 1972).

Lipids were extracted essentially by method B of Lloyd *et al.* (1972), dried at 40°C under N_2 , separated by two-dimensional t.l.c. (Yavin & Zutra, 1977) and visualized by exposure to iodine vapour and/or autoradiography. Spots corresponding to PtdIns and phosphatidate were scraped into vials and counted for radioactivity in a liquid-scintillation spectrometer.

Measurement of platelet cytosolic free Ca^{2+}

Platelet cytosolic free Ca^{2+} was monitored by using the fluorescent quinoline dye, quin 2, which

displays high affinity for Ca^{2+} and undergoes fluorescence changes resulting from Ca^{2+} binding (Tsien *et al.*, 1982). Platelet-rich plasma was incubated (37°C, 30 min) with quin 2-acetoxymethyl-ester which is membrane-permeant and crosses the plasma membrane, where it is rapidly hydrolysed by cytoplasmic esterases to the membrane-impermeant polycarboxylate anion that is quin 2. Quin 2 is thus trapped inside intact platelets and can report the resting concentration of cytosolic free Ca^{2+} and changes that result from exposure to agonists (Rink *et al.*, 1982). Quin 2-labelled platelets were separated from plasma containing extraneous dye by gel-filtration on columns of Sepharose 2B equilibrated with a modified Hepes-buffered Tyrodes' solution (129 mM-NaCl, 10.9 mM-trisodium citrate, 8.9 mM- NaHCO_3 , 0.56 mM-glucose, 5 mM-Hepes, 2.8 mM-KCl, 0.8 mM- KH_2PO_4 , 0.84 mM- MgCl_2 , 2.4 mM- CaCl_2 and 0.35% bovine serum albumin, pH 7.4). The quin 2 content of platelets was around 1 mmol/litre of cells. Gel-filtered platelets were stored at 37°C in stoppered plastic tubes, and, to compensate for the chelation of Ca^{2+} by citrate, the external free calcium concentration was adjusted to 1 mM by addition of CaCl_2 immediately before use. Platelets (1 ml) were placed in 1 cm square quartz cuvettes at 37°C, and fluorescence was monitored in an Aminco-Bowman spectrophotofluorimeter at 37°C with standard monochromator settings of 339 nm (excitation) and 500 nm (emission).

Platelet function tests

Platelet aggregation was measured photometrically in 0.17 ml samples of platelets. Release of platelet dense granule constituents (ADP, 5-hydroxytryptamine) was monitored by using an isotope (5-hydroxy[^{14}C]tryptamine) pre-labelling technique (MacIntyre *et al.*, 1978).

Other assays

Protein was estimated by the method of Lowry *et al.* (1951).

Materials

15(S)-Hydroxy-9 α ,11 α -epoxymethanoprostanoic acid (9 α ,11 α -epoxymethanoPGH $_2$), prostaglandin E_1 and prostaglandin I_2 were gifts from the Upjohn Co., Kalamazoo, MI, U.S.A. 9,11-Epoxymethano[^3H]PGH $_2$ (13.9 Ci/mmol) and ($\pm$)-5-endo-(6'-carboxyhex-2'-Z-enyl-6-exo-[N-(phenylcarbamoyl) hydrazonomethyl]-bicyclo[2,2,1]heptane (EP 045) were prepared as described previously (Wilson *et al.*, 1982; Armstrong *et al.*, 1983). 9,11-Epoxymethano[^3H]PGH $_2$ was dissolved in methanol. Prostaglandin I_2 was dissolved in Tris/HCl buffer, pH 9.2. Other prostaglandins were dissolved initially in ethanol and

diluted to 3.3 mM stock solutions by addition of 9 vol. of Na_2CO_3 (3 mM). Subsequent dilutions were made in iso-osmotic saline. Purified human fibrinogen was a gift from Dr. D. Lane, Charing Cross Hospital, London, U.K. Bovine serum albumin, and phospholipid carriers and standards were purchased from Sigma. T.l.c. plates (Macherey-Nagel; 'Polygram Sil G' pre-coated glass plates) were purchased from Camlab, Cambridge, U.K. All organic solvents and other laboratory reagents were analytical grade. Carrier-free $[^{32}\text{P}]\text{orthophosphate}$ was supplied by the Regional Isotope Dispensary, Western Infirmary, Glasgow, Scotland, U.K. 5-Hydroxy[*side-chain-2- ^{14}C*]tryptamine creatinine sulphate (53 mCi/mmol) was from Amersham International.

Results

Before attempting to correlate agonist-induced biochemical events to cellular responses it is mandatory to ensure that platelet functional responsiveness is maintained under the conditions used to monitor the biochemical events. Accordingly, we monitored the effects of 9,11-epoxymethanoPGH₂ on platelet aggregation using platelets prepared under conditions of $[^{32}\text{P}]\text{P}_i$ radiolabelling. Fig. 1 shows that 9,11-epoxymethano-

PGH₂ induces concentration-dependent platelet aggregation which is rapid and irreversible. Added fibrinogen (50 $\mu\text{g}/\text{ml}$) augments the aggregation responses but it is not a prerequisite for aggregation. Aggregation was not associated with significant release of the platelet dense granule marker, 5-hydroxy[^{14}C]tryptamine (results not shown). There is inter-subject variability in platelet responsiveness to 9,11-epoxymethanoPGH₂. The threshold concentration at which it induces platelet aggregation is around 10 nM, and maximum responses are observed at concentrations around 1 μM .

The optical techniques for monitoring platelet activation are acknowledged to be insensitive (Born & Hume, 1967). However, given the rapidity of onset of platelet aggregation induced by 9,11-epoxymethanoPGH₂ it is not unreasonable to assume that receptor-mediated transduction mechanisms should occur shortly after agonist addition. Fig. 2 depicts the time-course of the effects of 9,11-epoxymethanoPGH₂ (3.3 μM) on platelet $[^{32}\text{P}]\text{phosphatidate}$ and $[^{32}\text{P}]\text{PtdIns}$. Within 15 s of agonist addition, there is a rapid rise in $[^{32}\text{P}]\text{phosphatidate}$ to $344 \pm 41\%$ over control levels (mean $\pm$ s.d., $n = 3$). No further increases are observed between 15 and 30 s of agonist addition, and thereafter the levels of $[^{32}\text{P}]\text{phosphatidate}$

Fig. 1. 9,11-EpoxymethanoPGH₂-induced human platelet aggregation

Plasma-free suspensions of platelets were prepared under radiolabelling conditions (see the Experimental section) and fibrinogen (50 $\mu\text{g}/\text{ml}$) was added. Platelet aggregation was monitored photometrically in 0.17 ml samples following the addition (at the arrows) of 9,11-epoxymethanoPGH₂ at the concentrations indicated.

Fig. 2. Time course of $[^{32}\text{P}]$ phospholipid formation induced by 9,11-epoxymethanoPGH₂

Aliquots (0.4 ml) of ^{32}P -prelabelled platelets were incubated at 37°C in the presence of 9,11-epoxymethanoPGH₂ (3.3 μM) (closed symbols) or saline, in control samples (open symbols) for the time indicated. Reactions were quenched and phospholipids extracted, separated and the levels of $[^{32}\text{P}]$ phosphatidate (●, ○) and $[^{32}\text{P}]$ PtdIns (■, □) were monitored. Results shown are mean values $\pm$ S.E.M. of triplicate determinations. The experiment shown is typical of three similar experiments using platelets from different donors. Statistical analysis (control level versus exposure to 9,11-epoxymethanoPGH₂) was by Student's *t* test: *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 3. Concentration-response curves of 9,11-epoxymethanoPGH₂-induced $[^{32}\text{P}]$ phosphatidate formation

Aliquots (0.4 ml) of ^{32}P -prelabelled platelets were incubated at 37°C for 30 s in the presence of 9,11-epoxymethanoPGH₂ at the concentrations indicated (●) or saline, in control samples (○). Phospholipids were analysed as described in Fig. 2. Results shown are mean values $\pm$ S.E.M. of triplicate determinations. The experiments shown are typical of five similar experiments using platelets from different donors. Statistical analysis (control level versus exposure to 9,11-epoxymethanoPGH₂) was by Student's *t* test: *P < 0.05, ***P < 0.001.

decline to reach a steady, but significantly elevated, level. The levels of $[^{32}\text{P}]$ PtdIns did not change significantly compared with control levels until 60–120 s after agonist addition. At 600 s the $[^{32}\text{P}]$ PtdIns had increased to $144 \pm 19\%$ (mean $\pm$ S.D., *n* = 3) of the control level.

From the time-course experiments, peak stimulation of inositol lipid metabolism, as indicated by formation of $[^{32}\text{P}]$ phosphatidate, occurs within 15 s of agonist addition and does not decline until greater than 30 s of agonist addition. The concentration-response relationship for 9,11-epoxymethanoPGH₂-induced formation of $[^{32}\text{P}]$ phosphatidate, measured 30 s after agonist addition, is shown in Fig. 3. The threshold concentration of agonist is around 10–20 nM, and maximum stimulation of $[^{32}\text{P}]$ phosphatidate formation was observed at $[9,11\text{-epoxymethanoPGH}_2] = 330 \text{ nM}$ (*n* = 4) and at $[9,11\text{-epoxymethanoPGH}_2] = 1.1 \mu\text{M}$ (*n* = 3). The calculated EC₅₀ (the concentration of agonist that elicits 50% of the maximum response) is $54 \pm 8 \text{ nM}$ (mean $\pm$ S.D., *n* = 7).

Using platelets labelled with quin 2, the estimated resting cytosolic free Ca^{2+} concentration was $86 \pm 12 \text{ nM}$ (mean $\pm$ S.D., *n* = 6). 9,11-Epoxy-methanoPGH₂ produced a concentration-depend-

ent increase in the cytosolic free calcium concentration (Fig. 4). The changes in cytosolic free calcium induced by 9,11-epoxymethanoPGH₂ occurred rapidly following addition of agonist and were essentially complete within 10 s. The threshold concentration of 9,11-epoxymethanoPGH₂ at which a Ca^{2+} flux was observed was around 10–30 nM, and the maximum response was observed at $[9,11\text{-epoxymethanoPGH}_2] = 1 \mu\text{M}$. The estimated EC₅₀ was $80 \pm 23 \text{ nM}$ (mean $\pm$ S.D., *n* = 4). Quin 2 labelling of platelets did not impair functional responses or agonist-induced $[^{32}\text{P}]$ phosphatidate formation.

Human platelets contain a single class of stereospecific, saturable, high-affinity binding sites that have the pharmacological characteristics of a thromboxane A₂ receptor (Armstrong *et al.*, 1983). The relationship between thromboxane A₂ receptor occupancy and stimulation of inositol lipid

Fig. 4. Concentration-response curve of 9,11-epoxymethanoPGH₂-induced elevation of platelet intracellular free Ca²⁺ monitored as quin 2 fluorescence

Aliquots (1 ml) of quin 2-labelled platelets were incubated with 9,11-epoxymethanoPGH₂ at the concentrations indicated and fluorescence changes and intracellular free Ca²⁺ were monitored. Results shown are mean values $\pm$ S.D. of three to six observations using platelets obtained from different donors. Results are expressed as a percentage of the maximum response (increase in intracellular free Ca²⁺) which ranged from 206 to 603 nM.

metabolism was investigated by comparing the concentration-response relationships for 9,11-epoxymethano[³H]PGH₂ binding to its high-affinity binding site and 9,11-epoxymethanoPGH₂-induced [³²P]phosphatidate formation simultaneously using platelets isolated from the same donor. The cumulative results from four such experiments are shown in Fig. 5. There was no significant difference between the estimated K_D (concentration of radioligand at which 50% of the binding sites are occupied) for 9,11-epoxymethano[³H]PGH₂ binding of 70 ± 13 nM (mean $\pm$ S.D., $n = 4$) and the EC_{50} for stimulation of [³²P]phosphatidate formation by 9,11-epoxymethanoPGH₂ of 51 ± 9 nM (mean $\pm$ S.D., $n = 4$).

The compound EP 045 selectively inhibits platelet activation induced by thromboxane A₂ and thromboxane A₂ mimetics, and displaces 9,11-epoxymethano[³H]PGH₂ from its high-affinity binding site on platelets (Armstrong *et al.*, 1983). Thus EP 045 can be termed a thromboxane A₂ receptor antagonist. We therefore used EP 045 to confirm that 9,11-epoxymethanoPGH₂-induced [³²P]phosphatidate formation and calcium flux were receptor-mediated events. Fig. 6 shows the effects of EP 045 on [³²P]phosphatidate formation

Fig. 5. Concentration-dependence of 9,11-epoxymethanoPGH₂-induced [³²P]phosphatidate formation and receptor occupancy

Platelets were isolated from four different donors and each platelet sample was separated into two portions: one for measurement of [³²P]phosphatidate formation and the other for measurement of radioligand binding. For measurement of [³²P]phosphatidate formation, triplicate aliquots (0.4 ml) of ³²P-prelabelled platelets were incubated at 37°C for 30 s in the presence of 9,11-epoxymethanoPGH₂ at the concentrations indicated and [³²P]phosphatidate (○) was analysed as described in Fig. 2. For radioligand binding analysis, quadruplicate aliquots (1 ml) of platelets suspended in buffer were incubated for 4 min at 20°C with 9,11-epoxymethano[³H]PGH₂ at the concentrations indicated, in the presence or absence of excess unlabelled 9,11-epoxymethanoPGH₂ (11.4 μ M). Bound and free radioligand were separated by centrifugation (15000g, 2 min). By subtracting the amount of radioligand bound in the presence of excess unlabelled 9,11-epoxymethanoPGH₂ from that in its absence, specific (i.e. displaceable) binding was determined (●) (Armstrong *et al.*, 1983). To facilitate comparison, the data from each experiment were expressed as percentages of the maximum response elicited in that preparation. The results shown are mean values ($\pm$ S.D.) of the observations obtained in the four different platelet samples. Maximum [³²P]phosphatidate formation ranged from 810 to 1570 c.p.m./mg of protein, and maximum specifically bound 9,11-epoxymethano[³H]PGH₂ ranged from 123 to 419 fmol/assay.

induced by 9,11-epoxymethanoPGH₂ (330 nM). At [EP 045] = 10 μ M, the response to the agonist is abolished, and the I_{50} value for EP 045 (concentration of antagonist that reduces the agonist response by 50%) was 450 ± 140 nM (mean $\pm$ S.D., $n = 3$). To confirm that the effect of EP 045 was selective for 9,11-epoxymethanoPGH₂, we examined its effects on [³²P]phosphatidate formation induced by other agonists, platelet-activating factor and vasopressin

Fig. 6. Effect of EP 045 on 9,11-epoxymethanoPGH₂-induced [³²P]phosphatidate formation by human platelets. Aliquots (0.4 ml) of ³²P-prelabelled platelets were incubated for 60 s at 37°C with saline (open symbols) or EP 045 at the concentrations indicated (closed symbols) prior to the addition of saline (Δ, ▲) or 330 nM-U44069 (○, ●) for a further 30 s. [³²P]Phosphatidate was analysed as described in Fig. 2. Results are mean values ± S.E.M. of triplicate observations. The experiment shown is typical of three similar experiments using platelets from different donors. Statistical analysis (9,11-epoxymethanoPGH₂ alone versus 9,11-epoxymethanoPGH₂ in the presence of EP 045) was by Student's *t* test: **P < 0.01, ***P < 0.001.

(MacIntyre & Pollock, 1982). In these experiments, to eliminate any contribution from endogenous arachidonate metabolites, platelets were obtained from aspirin-treated donors. EP 045 (3 μM) abolished [³²P]phosphatidate formation induced by 9,11-epoxymethanoPGH₂ (100 nM) but had no effect on submaximal responses to vasopressin (100 nM) and platelet-activating factor (100 nM).

When tested against 9,11-epoxymethanoPGH₂-induced increase in platelet cytosolic Ca^{2+} , EP 045 exerted a concentration-dependent inhibitory effect (Fig. 7). This effect of EP 045 was selective for 9,11-epoxymethanoPGH₂ in that responses of similar magnitude induced by platelet-activating factor were unaffected by EP 045 (<10 μM) (results not shown).

Discussion

The involvement of intracellular Ca^{2+} concentration as a major determinant of platelet activation derives from studies using calcium ionophores (Feinstein & Fraser, 1975), ⁴⁵Ca influx (Owen & Le Breton, 1981), permeabilized cells (Knight & Scrutton, 1980) and latterly, direct estimation of platelet cytosolic free Ca^{2+} concentration (Rink *et al.*, 1981, 1982). Specifically, the role of Ca^{2+} as a mediator of the effects of prostaglandin endoperoxides and thromboxane A₂ on platelets stemmed from the observations by Gerrard *et al.* (1976, 1978) that arachidonic acid metabolism occurred in the platelet dense tubular system, a calcium storage organelle, and that prostaglandins G₂, H₂ and thromboxane A₂ could promote Ca^{2+} release from this organelle into the platelet cytoplasm. Such cal-

Fig. 7. Effect of EP 045 on 9,11-epoxymethanoPGH₂-induced changes in the fluorescence of quin 2-loaded platelets. Plasma-free suspensions of quin 2-labelled platelets in a modified HEPES-buffered Tyrodes solution containing 1 mM-external Ca^{2+} were prepared as described in the Experimental section. Platelets were incubated for 60 s at 37°C with EP 045 at the concentration indicated or saline in control samples, and the fluorescence responses (excitation 339 nm, emission 500 nm) of 1 ml samples were monitored following the addition of 100 nM-9,11-epoxymethanoPGH₂. The appropriate intracellular free Ca^{2+} calibration scale is shown at the right of the fluorescence records. The data shown are typical of two similar experiments using platelets from different donors.

cium-releasing activity of endogenous prostaglandin endoperoxides and thromboxane A_2 may not be shared by exogenous arachidonate metabolites. Indeed, there is compelling evidence that exogenous prostaglandins G_2 , H_2 and thromboxane A_2 induce platelet activation by combining with distinct (thromboxane A_2) receptors on the platelet plasma membrane (MacIntyre, 1981; Armstrong *et al.*, 1983).

The nature of the coupling mechanisms that link thromboxane A_2 receptor occupancy to platelet activation are not fully understood. In view of the rapid bio-conversion of prostaglandins G_2 and H_2 and the instability of thromboxane A_2 , such mechanistic studies can best be performed using stable prostaglandin analogues that act as thromboxane A_2 mimetics in a variety of biological preparations (Coleman *et al.*, 1981; MacIntyre, 1981). Such studies indicate that platelet thromboxane A_2 receptor occupancy is not linked to adenylate cyclase or guanylate cyclase nor to acyl hydrolase activation (Best *et al.*, 1979; Di Minno *et al.*, 1981). As inositol phospholipid metabolism may be the receptor-mediated event that initiates Ca^{2+} flux in a number of systems, we examined the inter-relationships between thromboxane A_2 receptor occupancy, inositol phospholipid metabolism, cytosolic free Ca^{2+} concentration and human platelet activation.

Phospholipid analyses were performed using platelets that had been incubated with [^{32}P]P, until incorporation of radiolabel into phosphatidate had reached steady-state. Thus changes in labelling of [^{32}P]phosphatidate should reflect changes in phosphatidate concentrations. Under these conditions, it has been shown that agonist-induced formation of [^{32}P]phosphatidate by human platelets (Siess *et al.*, 1983) is comparable in magnitude to the change in cellular content of phosphatidate (Broekman *et al.*, 1980). A similar strategy has been used by Weiss *et al.* (1982) to monitor secretagogue-induced phosphatidate synthesis in parotid acinar cells. Although recent evidence implicates polyphosphoinositide metabolism rather than PtdIns metabolism as the initial event that follows occupancy of Ca^{2+} -mobilizing receptors in several systems (Kirk, 1982; Berridge, 1983; Drummond *et al.*, 1984), it should be noted that whether PtdIns, PtdIns(4)P or PtdIns(4,5)P₂ is/are hydrolysed by a phospholipase C type enzyme, one of the initial products is 1,2-diacylglycerol which would be rapidly converted to phosphatidate by diacylglycerol kinase. Clearly, agonist-induced formation of [^{32}P]phosphatidate could occur by mechanisms other than degradation of inositol phospholipids, for example (a) synthesis *de novo* from glycerol-3-phosphate; (b) phosphorylation of diacylglycerol derived from other phospholipids; (c)

phosphorylation of diacylglycerol derived from triacylglycerol. We can preclude these alternative sources of agonist-induced phosphatidate formation as (a) synthesis *de novo* of phosphatidate, even in stimulated platelets, is relatively slow (Neufeld & Majerus, 1983); (b) human platelet phospholipase C shows an absolute specificity for inositol phospholipids (Rittenhouse-Simmons, 1979); (c) human platelets contain negligible triacylglycerol lipase activity (Lote & Lowery, 1979).

The thromboxane A_2 mimetic, 9,11-epoxy-methanoPGH₂, stimulates, in a concentration-dependent manner, human platelet activation, formation of [^{32}P]phosphatidate and an elevation of the platelet cytosolic free Ca^{2+} concentration. As these events can occur in the absence of detectable platelet degranulation and in aspirin-treated platelets, they are independent of endogenous platelet agonists and must be mediated directly. The temporal relationship between the various events is such that within 15 s of agonist addition, the earliest time point at which phospholipid analyses could be performed routinely, platelet activation is under way and Ca^{2+} flux and [^{32}P]phosphatidate formation are maximal. Changes in labelling of [^{32}P]PtdIns are only observed following prolonged (>2 min) exposure to agonist. This presumably reflects the slower conversion of [^{32}P]phosphatidate to [^{32}P]PtdIns.

The thromboxane A_2 receptor antagonist EP 045 selectively and potently inhibits 9,11-epoxy-methanoPGH₂-induced platelet activation, Ca^{2+} flux and [^{32}P]phosphatidate formation, indicating that all such events are sequelae of thromboxane A_2 receptor occupancy. The lack of availability of sufficient quantities of fresh platelets only permitted the simultaneous comparison of [^{32}P]phosphatidate formation to receptor occupancy in any single platelet preparation. When the data are scaled to the same maximum, the isotherm for agonist binding (receptor occupancy) is similar to the concentration-response curves of [^{32}P]phosphatidate formation (Fig. 3) and of elevation of intracellular Ca^{2+} (Fig. 4). Thus, maximum phosphatidate formation and elevation of cytosolic Ca^{2+} are evoked when all the thromboxane A_2 receptors are occupied by 9,11-epoxymethanoPGH₂, indicating that there are few spare receptors (or little 'receptor reserve') in the system. In this respect, 9,11-epoxymethanoPGH₂-induced platelet activation resembles peptidergic and α -adrenergic stimulation of parotid acinar cells (Weiss & Putney, 1981). It should be noted that this assessment of the receptor reserve for the natural agonist, thromboxane A_2 , may be an underestimate should 9,11-epoxymethanoPGH₂ act as a partial agonist on platelets as on thromboxane A_2 -sensitive smooth muscle preparations (Jones *et al.*,

1982). Because of the insensitivity of optical techniques for monitoring platelet activation, any comparison of the concentration-response relationship between platelet activation and the other parameters under investigation must be made with caution. Nevertheless, it is of interest that 9,11-epoxymethanoPGH₂-induced platelet shape change and aggregation occurs over the same agonist concentration range as does increased Ca^{2+} availability and phosphatidate formation.

The observed stimulation of [^{32}P]phosphatidate formation by 9,11-epoxymethanoPGH₂ is apparently at variance with the findings of Rittenhouse & Allen (1982). These workers, using unlabelled platelets or [^3H]arachidonate-labelled platelets, reported that neither prostaglandin H₂ nor a stable analogue, 11,9-epoxymethanoPGH₂, was by itself an efficient stimulus of PtdIns metabolism. However, both thromboxane A₂ mimetics enhanced collagen-induced PtdIns metabolism. The reasons for this discrepancy are not obvious. It is probable that [^{32}P]P_i labelling yields a more sensitive index of phosphatidate formation than does [^3H]arachidonate labelling (cf. MacIntyre & Pollock, 1983), or phosphorus determinations. The present studies were performed upon platelets within 3–4 h of venepuncture, whereas Rittenhouse & Allen (1982) used platelets obtained within 15 h of venepuncture. As platelet responsiveness to thromboxane A₂ mimetics declines with time interval after venepuncture (Di Minno *et al.*, 1981), the most likely explanation of the discrepancy is that in the latter situation the platelets utilized were unresponsive (or desensitized) to thromboxane A₂ mimetics, unless primed by exposure to a different agonist, namely collagen.

Our findings indicate that human platelet thromboxane A₂ receptor occupancy is closely linked to inositol phospholipid metabolism and to Ca^{2+} flux. However, the inter-relationships between inositol phospholipid metabolism, Ca^{2+} flux and platelet activation remain to be established. Rink *et al.* (1982), using ionomycin, have reported that the threshold concentrations of cytosolic free Ca^{2+} necessary for platelet shape-change and aggregation are respectively 500 and 2000 nM. In the present study, maximum platelet aggregation in response to 9,11-epoxymethanoPGH₂ occurred when the cytosolic free Ca^{2+} concentration was below 700 nM. Thus an elevation in the cytosolic free Ca^{2+} concentration *per se* cannot explain platelet activation induced by thromboxane A₂ mimetics. Although agonist-induced breakdown of inositol phospholipids may be the link between receptor occupancy and Ca^{2+} flux (Michell, 1975; Berridge, 1981; Putney, 1981) both events may contribute to the platelet activation process. It is probable that Ca^{2+} and the immediate precursor of

phosphatidate, diacylglycerol (via activation of protein kinase C) may act synergistically or even synarchically (Rasmussen, 1981; Kaibuchi *et al.*, 1983; Michell, 1983) to mediate the cellular responses to thromboxane A₂ receptor occupancy.

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