Liberation of [³H]arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen

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Cytosolic Ca²⁺ levels and arachidonate liberation were investigated in platelets loaded with the fluorescent Ca²⁺ indicator dye fura-2, and labelled with [³H]arachidonate. Fura-2 was used in preference to quin2 because the latter interfered with [³H]arachidonate labelling of phospholipids. 2. From a resting free Ca²⁺ level of around 100 nM, ionomycin (10–200 nM) evoked an instantaneous, concentration-dependent increase in cytosolic Ca²⁺ that only resulted in [³H]arachidonate liberation (up to 4-fold over control) at Ca²⁺ levels > 1 μM. 3. Addition of collagen (10 μg/ml) evoked an elevation in Ca²⁺ up to 461 ± 133 nM. These changes in Ca²⁺ were accompanied by a 2–4-fold elevation in [³H]arachidonate with depletion of [³H]phosphatidylcholine by 17 ± 4% and [³H]phosphatidylinositol by 41 ± 7%. 4. Indomethacin (10 μM) reduced the elevation in Ca²⁺ by collagen to 115 ± 18 nM but did not significantly inhibit the 2–4-fold increase in [³H]arachidonate. [³H]Phosphatidylcholine and [³H]phosphatidylinositol were decreased by 9 ± 7% and 10 ± 6%, respectively, with collagen in the presence of indomethacin. 5. Stimulation of phosphoinositide turnover by collagen in the presence and absence of indomethacin was indicated by [³²P]phosphatidate formation in cells prelabelled with [³²P]P_i. This phosphatidate formation was decreased (75%) by the presence of indomethacin. 6. In the presence of indomethacin, phorbol myristate acetate (20 nM) alone or in combination with ionomycin (30 nM) failed to stimulate arachidonate liberation despite a marked stimulation of aggregation. 7. These results indicate that, whereas ionomycin requires Ca²⁺ in the μM range for arachidonate liberation, collagen, notably in the presence of indomethacin, does so at basal Ca²⁺ levels. The mechanisms underlying the regulation of arachidonate release by collagen are not clear, but do not appear to involve activation of protein kinase C, or an elevation of cytosolic free Ca²⁺.

INTRODUCTION

In response to certain physiological or pharmacological stimuli many different cell types synthesize and release by exocytosis one or more of the biologically active lipids that are derived from arachidonic acid. These lipids, collectively known as eicosanoids (Corey *et al.*, 1980), include prostaglandins, thromboxanes and leukotrienes. Because the intracellular level of free arachidonate is low in most cells, the rate-determining step in eicosanoid biosynthesis is usually the liberation of free arachidonate from glycerolipids (for review see Irvine, 1982).

The mechanisms underlying arachidonic acid liberation in platelets have been extensively investigated. In these cells arachidonic acid is predominantly found esterified in the *sn*-2 position of membrane phospholipids, particularly PtdCho, PtdEtn and PtdIns. Four major routes for arachidonate liberation have been proposed: (i) direct deacylation of phospholipids by a phospholipase (PL) A₂ type enzyme (McKean *et al.*, 1981); (ii) deacylation by a PLA₁ activity followed by a lysophospholipase; (iii) sequential activation of phosphoinositide-specific PLC followed by diacylglycerol lipase (Mauco *et al.*, 1978; Rittenhouse-Simmons, 1979; Bell *et al.*, 1979); (iv)

phosphatidate-specific PLA₂ following phosphoinositide-specific PLC/diacylglycerol kinase (Billah *et al.*, 1981).

Phospholipases of the A₂ type have been considered to be Ca²⁺-calmodulin-dependent and as such susceptible to activation following an increase in [Ca²⁺]_i (Wong & Cheung, 1979; but see also Withnall *et al.*, 1984; Ballou & Cheung, 1985). In contrast, activation of phosphoinositide-specific PLC as a consequence of specific agonist-receptor interactions is Ca²⁺-independent and results in the generation of intracellular second messengers including an elevation in [Ca²⁺]_i (Berridge, 1984) which may then activate Ca²⁺-calmodulin-dependent processes. These inositide-specific reactions may be relevant to receptor-mediated liberation of free arachidonate as proposed in routes (iii) and (iv) above; the question remains, however, as to whether receptor occupation itself, or a subsequent elevation in [Ca²⁺]_i, is the controlling factor in evoking free arachidonate liberation.

Activation of blood platelets by collagen results in stimulation of phosphoinositide metabolism (Lloyd *et al.*, 1973; Rittenhouse-Simmons & Allen, 1982; Watson *et al.*, 1985), an elevation in [Ca²⁺]_i (Rink & Sanchez, 1984) and specific protein phosphorylation (Sano *et al.*, 1983).

Abbreviations used: PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdA, phosphatidic acid; InsP₃, inositol 1,4,5-trisphosphate; Tx, thromboxane; PMA, phorbol myristate acetate; PL, phospholipase.

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However, both these biochemical and functional responses are markedly attenuated by cyclo-oxygenase blockade with aspirin or indomethacin, indicating a pivotal role for TxA_2 in mediating these effects of collagen. Thromboxane receptors on human platelets are coupled to stimulation of phospho-inositide metabolism and an elevation in $[\text{Ca}^{2+}]_i$ (Pollock *et al.*, 1984) and so it is likely that many of the biochemical responses observed in response to collagen are mediated by prostaglandin endoperoxides and/or TxA_2 (Siess *et al.*, 1983). Thus the paradox remains as to how the free arachidonate necessary for TxA_2 synthesis is liberated during the initial platelet-collagen interaction.

To what extent PLA or PLC are regulated by Ca^{2+} has been confused by the examination of these enzymes under non-physiological conditions using 'artificial' substrates and/or 'artificial' environments (for a discussion of these, see for example Irvine *et al.*, 1984a). The development of the fluorescent Ca^{2+} indicator dye quin2 (Tsien *et al.*, 1982) has allowed $[\text{Ca}^{2+}]_i$ measurements in intact platelets at rest or during stimulation by agonists (Tsien *et al.*, 1984). Recently a novel fluorescent Ca^{2+} -indicator dye, fura-2, has been developed by Tsien and colleagues (Grynkiewicz *et al.*, 1985). Fura-2 exhibits much greater fluorescence than quin2 and so it may be used at much lower loading concentrations in the cell. Using fura-2-loaded platelets to monitor $[\text{Ca}^{2+}]_i$ we have investigated the regulation of arachidonate liberation in response to collagen and the Ca^{2+} ionophore ionomycin. In the present paper we present evidence to suggest a differential Ca^{2+} -sensitivity for the liberation of arachidonate in human platelets in response to these two agonists.

EXPERIMENTAL

Materials

Soluble collagen reagent (Horm) was from Malin Medical Supplies, Alloway, Scotland, U.K.; ionomycin (Calbiochem), fura-2 acetoxymethyl ester (a gift from Dr Roger Tsien, University of California, Berkeley, CA, U.S.A. or from Molecular Probes, Junction City, OR, U.S.A.), PMA (Sigma) and digitonin (BDH) were dissolved in dimethyl sulphoxide. Indomethacin (Sigma) was dissolved at a concentration of 10 mM in methanol. Lipid standards were prepared from yeast and pig liver; PtdA was prepared from yeast PtdCho by PLD activity from a cauliflower extract (Dawson & Hemington, 1967). All other laboratory reagents and solvents were of analytical grade. T.l.c. plates (Merck silica gel 60 F-254 precoated glass plates) were from Camlab, Cambridge, U.K.

[5,6,8,9,11,12,14,15- ^3H]Arachidonic acid (80–135 Ci/mmol) and carrier-free [^{32}P]P_i were from Amersham International, Amersham, Bucks, U.K.

Preparations of cells

Blood was obtained by venepuncture from healthy human volunteers, who denied taking aspirin in the previous 10 days, and was mixed with one-sixth volume of acid citrate dextrose (2.5 g of sodium citrate, 1.5 g of citric acid and 2 g of D-glucose in 100 ml of water). This blood was then centrifuged (700 g, 5 min 15–20 °C) and the supernatant (platelet-rich plasma) aspirated into

plastic tubes. Plasma-free suspensions of platelets were then prepared by centrifugation (350 g, 20 min, 15–20 °C) of the platelet-rich plasma in the presence of 20 µg of apyrase/ml, discarding the supernatant, and resuspending the cells in physiological saline (145 mM-NaCl, 5 mM-KCl, 1 mM-MgSO₄, 10 mM-Hepes and 10 mM-glucose, pH 7.4 at 37 °C) at 3×10^8 cells/ml. The platelet suspension was then incubated for 45 min at 37 °C in the presence of 1 µM of the membrane-permeant fura-2 acetoxymethyl ester and 2 µCi of [^3H]arachidonic acid/ml. In fura-2-loaded platelets the maximum fluorescence (F_{max}) is equivalent to 3 times the cell autofluorescence when the loading concentration of fura-2 is 30 µM (T. J. Rink & R. Y. Tsien, unpublished work). The fura-2 loading concentration using the above protocol was calculated to be 20–30 µmol/litre of cell water. In some experiments 20 µCi of [^{32}P]P_i was also present. After loading and labelling, the pH of the cell suspension was adjusted to < 6.5 by the addition of 0.2% (v/v) acid citrate dextrose. Platelets were then pelleted by centrifugation (350 g, 20 min, 15–20 °C) in the presence of 20 µg of apyrase/ml, resuspended in fresh buffer at $(1.5\text{--}2) \times 10^8$ cells/ml and stored at room temperature in a stoppered plastic tube until use. In preliminary experiments to determine the effect of quin2 loading on [^3H]arachidonate incorporation, platelet-rich plasma was incubated with 2 µCi of [^3H]arachidonic acid/ml in the absence (control) or presence (quin2 loading) of 15 µM-quin2 acetoxymethyl ester, for 30 min at 37 °C. These conditions give 1–1.5 mM internal quin2.

Experimental protocol

Aliquots (0.8 ml) of cells were dispensed into round bottom test tubes and the external Ca^{2+} ($[\text{Ca}^{2+}]_o$) concentration adjusted to 1 mM with CaCl_2 . These samples were then transferred to a purpose-built cuvette holder and thermostatted at 37 °C in a Perkin-Elmer MPF 44A spectrophotometer which allowed simultaneous measurement of fluorescence and absorbance. The cell suspension was continuously stirred with a magnetic follower so that platelet shape change/aggregation could be monitored turbidimetrically. Following equilibration at 37 °C for at least 5 min, agonists were added to the platelets as indicated. At the appropriate time after agonist addition the reaction was stopped by the addition of 1 ml of ice-cold methanol to the cells and the entire sample was transferred by plastic pipette to a Pyrex test tube on ice. Within any one experiment duplicate or triplicate determinations of each agonist addition were carried out. To minimize time-dependent effects on platelet responsiveness, leakage of the dye or redistribution of radiolabel, experiments were designed to be completed within 45 min–1 h.

Measurement of cytosolic free calcium levels

In an analogous manner to quin2, fura-2 is liberated from its methyl ester by cytosolic esterases inside the cell. Upon binding of Ca^{2+} , fura-2 undergoes both a change in fluorescence intensity and a spectral shift (Grynkiewicz *et al.*, 1985). For our purposes fura-2 fluorescence was monitored continuously using monochromator settings of 339 nm (excitation) and 500 nm (emission). The $[\text{Ca}^{2+}]_i$ levels were calculated using the general formula

$$\text{Ca}^{2+} = K_d(F - F_{\text{min.}} / (F_{\text{max.}} - F))$$

where K_d is the dissociation constant for Ca^{2+} binding to the indicator and F is arbitrary fluorescent units. For fura-2, $K_d = 224 \text{ nM}$ (Grynkiewicz *et al.*, 1985). F_{max} was determined by lysing the cells with $50 \mu\text{M}$ -digitonin, thus exposing the fura-2 to 1 mM - Ca^{2+} . F_{min} was determined by adjusting the pH of the lysed cells to 8.5 with 20 mM -Tris base followed by the addition of 10 mM -EGTA. Under these conditions, the K_d for Ca^{2+} -EGTA is $< 10^{-9} \text{ M}$. Alternatively, by quenching the fluorescence with 2 mM - Mn^{2+} to give the autofluorescence (AF) of the cells, F_{min} can be calculated from the relationship between F_{max} , F_{min} , and AF as derived from the emission spectra of Ca^{2+} -saturated and Ca^{2+} -free dye (Grynkiewicz *et al.*, 1985; Tsien *et al.*, 1985). For our spectrophotometer, using glass cuvettes at the monochromator settings used, we calculated this relationship to be:

$$F_{\text{min}} = AF + (F_{\text{max}} - AF)/3.7$$

Lipid analyses

Lipids were extracted essentially according to Bligh & Dyer (1959) by the addition of 1.5 ml of chloroform/methanol (2:1, v/v) plus $20 \mu\text{l}$ of 10 M -HCl to the quenched platelets. After vortex-mixing, the aqueous and organic phases were partitioned by the addition of 1 ml of chloroform, followed by 1 ml of water, and separated by centrifugation ($1000 g$ for 5 min). The organic (lower) phase was removed into a glass test tube with a pasteur pipette and dried down at 40°C under a stream of N_2 .

Separation by t.l.c.

Major phospholipids and free fatty acids, including arachidonate, were separated by one-dimensional t.l.c. using a system modified from that of Irvine *et al.* (1984b). T.l.c. plates were sprayed with 1 mM -EDTA (adjusted to pH 5.5 with NaOH) until saturated, and were then activated for 1 h at 160°C in an oven. The lipids were resuspended to $100 \mu\text{l}$ in chloroform/methanol (9:1, v/v) and spotted, using a Hamilton syringe, as 1.5 cm bands 1.5 cm from the bottom of the plate. To facilitate visualization of the lipids with iodine, $5\text{--}10 \mu\text{g}$ of carrier PtdIns, PtdA and oleic acid were added to each lipid sample. The t.l.c. plates were developed in one dimension for 1 h using a solvent of chloroform/methanol/water/acetic acid (65:50:5:2, by vol.) until the solvent front was $12\text{--}14 \text{ cm}$ from the origin. Individual lipids were visualized by exposure of the plate to iodine vapour and identified by co-migration with lipid standards. This separation resolved PtdCho, PtdSer, PtdIns, PtdA, PtdEtn and free fatty acids including arachidonate. Silica corresponding to these lipids was scraped into a 4 ml plastic scintillation vial containing 3.5 ml of scintillation fluid and the samples were counted in a liquid-scintillation spectrometer.

Statistical analysis of results

Results are expressed as mean values of n determinations $\pm$ S.D. or S.E.M. Statistical comparison of control additions versus agonist additions was by Student's t test with P values of $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

RESULTS

Effect of quin2 and fura-2 on [^3H]arachidonate incorporation

At the commencement of our studies we had at our disposal both quin2 and fura-2 acetoxymethyl esters. In view of several reports of toxic effects of quin2 (Rink & Pozzan, 1985), perhaps as a result of the high intracellular loading concentration, we investigated the effects of quin2 and fura-2 loading on the incorporation of [^3H]arachidonate into platelet phospholipids. We labelled platelets with [^3H]arachidonate in platelet-rich plasma in the absence or presence of quin2 acetoxymethyl ester, and then resuspended the platelets in buffer; [^3H]phospholipids were analysed from duplicate 0.8 ml aliquots of cells. In four paired trials, quin2 loading suppressed [^3H]PtdCho labelling by 35% , enhanced [^3H]PtdIns labelling by 50% , and had little effect on [^3H]PtdA labelling. Similar effects were observed when [^3H]arachidonate was incorporated into resuspended platelets preloaded with quin2. In contrast, platelets loaded with fura-2 showed no significant difference in [^3H]arachidonate incorporation into phospholipids when compared with control unloaded platelets. Furthermore, direct comparison of loaded with unloaded platelets with regard to the effects of ionomycin and collagen showed that fura-2 loading has no effect on the phenomena shown in this paper (see below for details). Whatever the mechanisms for the differences between fura-2 and quin2, we assume that these result from the much lower loading used with fura-2, although we have not specifically investigated this possibility. Fura-2 certainly appears to have significant advantages over quin2 for our investigative purposes and was therefore used in all our studies.

Changes in $[\text{Ca}^{2+}]_i$ and platelet lipids in response to ionomycin

The Ca^{2+} ionophore ionomycin provides a pharmacological tool with which to evoke changes in $[\text{Ca}^{2+}]_i$ while bypassing the receptor-operated mechanisms. Resting control $[\text{Ca}^{2+}]_i$ levels and the effects upon this of low concentrations of ionomycin ($10\text{--}100 \text{ nM}$) in the presence of extracellular Ca^{2+} at 1 mM are illustrated in Fig. 1. The resting $[\text{Ca}^{2+}]_i$ level of around 100 nM (Fig. 1a) in fura-2-loaded platelets is similar in magnitude to that previously reported using quin2-loaded cells. Ionomycin induced a rapid, concentration-dependent elevation in $[\text{Ca}^{2+}]_i$ as shown in Fig. 1. The reactions were quenched 150 s after control or agonist additions and the platelet lipids were analysed. In Table 1 the calculated mean maximum $[\text{Ca}^{2+}]_i$ levels depicted in Fig. 1, are summarized, as are the levels of [^3H]lipids. Ionomycin at 100 nM or lower failed to produce any statistically significant changes in any of the lipids examined, namely [^3H]PtdCho, [^3H]PtdIns and [^3H]arachidonate. This lack of effect was also observed in three similar experiments where ionomycin produced an increase in $[\text{Ca}^{2+}]_i$ to $< 1 \mu\text{M}$. However, an elevation in $[\text{Ca}^{2+}]_i$ to $> 1 \mu\text{M}$ using high concentrations ($100\text{--}200 \text{ nM}$) of ionomycin resulted in substantial liberation of free [^3H]arachidonate which is indicated in Fig. 2; this was accompanied by significant depletion in [^3H]PtdCho (results not shown). To prevent positive feedback from the endogenous generation of TxA_2 by the platelets, the experiments employing high concentrations of ionomycin were carried out in the presence of indomethacin ($10 \mu\text{M}$). Some experiments

Fig. 1. Changes in platelet $[Ca^{2+}]_i$ in response to ionomycin

In the presence of 1 mM- $[Ca^{2+}]_o$, ionomycin at the concentrations indicated, or dimethyl sulphoxide (DMSO) (1 μ l) in control samples, was added to 0.8 ml suspensions of fura-2 loaded and $[^3H]$ arachidonate-labelled platelets at 37 °C, prepared as described in the Experimental section. At 150 s after agonist additions the reactions were quenched with ice-cold methanol. The fluorescence calibration for $[Ca^{2+}]_i$ was calculated as outlined in the Experimental section. The Figure shows representative traces from triplicate determinations.

using low concentrations of ionomycin were also performed in the presence of indomethacin where indicated in Fig. 2. By plotting the $[Ca^{2+}]_i$ versus accumulation of free $[^3H]$ arachidonate, the Ca^{2+} -dependency curve for the liberation of arachidonate in response to ionomycin is obtained (Fig. 2).

Changes in $[Ca^{2+}]_i$ and platelet lipids in response to collagen

In contrast with ionomycin, collagen (Fig. 3b) added to fura-2-loaded platelets had no effect during a 10–20 s lag phase, but then caused a rise in $[Ca^{2+}]_i$ from around 100 nM to around 500 nM. In parallel with the collagen-induced $[Ca^{2+}]_i$ changes, the absorbance changes in Fig. 3(b) indicate that the platelets undergo shape change 10–20 s after collagen addition, and this is rapidly followed by platelet aggregation. The onset of aggregation causes a problem when monitoring fluorescence changes, because the formation of platelet aggregates quenches the fluorescence signal. Hence, although the declining phase of the fluorescence record in Fig. 3(b) could in part initially reflect Ca^{2+} sequestration/extrusion, the rapidly oscillating decline in the signal is mostly due to quenching

Fig. 2. Correlation between changes in platelet $[Ca^{2+}]_i$ and $[^3H]$ arachidonate liberation in the presence of ionomycin or collagen

The percentage stimulation of $[^3H]$ arachidonate levels over controls was plotted against the corresponding $[Ca^{2+}]_i$ following stimulation of fura-2-loaded and $[^3H]$ arachidonate-labelled platelets with collagen (10 μ g/ml) (squares) or ionomycin (circles) in the presence (open symbols) or absence (closed symbols) of 5 min prior to the agonist. The data are pooled from several experiments using platelets from different donors, and each point represents the mean value of a duplicate or triplicate determination. Control $[^3H]$ arachidonate levels ranged from 3000 to 8000 c.p.m./ 2×10^8 cells; resting $[Ca^{2+}]_i$ levels ranged from 90 to 120 nM.

by platelet aggregates. It is therefore probable that the maximum observed elevation in $[Ca^{2+}]_i$ in response to collagen is to some extent underestimated. However, in seven experiments using platelets from different donors the observed mean maximum rise in $[Ca^{2+}]_i$ in response to 10 μ g of collagen/ml was 461 ± 133 nM ($\pm$ S.D.).

The contribution of cyclo-oxygenase products in mediating the effects of collagen was investigated by using indomethacin. Changes in $[Ca^{2+}]_i$ and platelet shape-change/aggregation in response to 10 μ g of collagen/ml in the presence of 10 μ M-indomethacin are shown in Fig. 3(c). In this particular experiment the maximum $[Ca^{2+}]_i$ level reached after stimulation by collagen in the presence

Table 1. Platelet lipid changes associated with ionomycin-induced changes in $[Ca^{2+}]_i$

Following quenching, the platelet samples from Fig. 1 were extracted and the lipids analysed following separation by t.l.c. $[Ca^{2+}]_i$ values were calculated from the calibration in Fig. 1. The results shown are mean values $\pm$ S.D., of triplicate determinations from one of three similar experiments.

Additions	Maximum $[Ca^{2+}]_i$ (nM)	$[^3H]$ Lipids (c.p.m./ 2×10^8 cells)		
		Arachidonic acid	PtdCho	PtdIns
Controls	107 ± 7	4693 ± 539	$81\,699 \pm 829$	$20\,724 \pm 842$
Ionomycin (10 nM)	240 ± 30	4666 ± 82	$85\,544 \pm 2169$	$19\,582 \pm 1953$
Ionomycin (60 nM)	345 ± 85	4610 ± 599	$81\,900 \pm 1058$	$20\,359 \pm 246$
Ionomycin (100 nM)	809 ± 257	5127 ± 1115	$80\,129 \pm 2050$	$19\,385 \pm 649$

Fig. 3. Effect of collagen on platelet $[\text{Ca}^{2+}]_i$ and platelet shape change/aggregation

Additions: (a) control, (b) collagen (10 $\mu\text{g}/\text{ml}$) and (c) collagen (10 $\mu\text{g}/\text{ml}$) in the presence of 10 μM -indomethacin, added 5 min prior to the agonist, were made as described for Fig. 1. Fluorescence and absorbance changes were monitored simultaneously. The fluorescence calibration for $[\text{Ca}^{2+}]_i$ was calculated as outlined in the Experimental section. The traces shown are representative of triplicate determinations from one of four similar experiments.

of indomethacin was $123 \pm 19 \text{ nM}$ ($\pm \text{s.d.}$, $n = 3$), a value that is not statistically different from resting levels of $115 \pm 18 \text{ nM}$ ($\pm \text{s.d.}$, $n = 12$). Platelet shape change was abolished by indomethacin, and platelet aggregation was markedly inhibited; thus the problems caused by aggregation of platelets interfering with fluorescence measurements (discussed above) do not arise. In four similar experiments indomethacin reduced the maximum

observed rise in $[\text{Ca}^{2+}]_i$ in response to collagen from approx. 425 ± 130 to $125 \pm 22 \text{ nM}$ ($\pm \text{s.d.}$).

The lipid changes associated with the $[\text{Ca}^{2+}]_i$ responses in Fig. 3 are summarized in Table 2. In both the absence and presence of indomethacin, collagen caused a 4-fold stimulation in free $[^3\text{H}]$ arachidonate levels. In several experiments the stimulation of free $[^3\text{H}]$ arachidonate ranged from 2–4-fold over control levels. These results are depicted in Fig. 2 where the correlation between $[\text{Ca}^{2+}]_i$ and free $[^3\text{H}]$ arachidonate accumulation in response to collagen/indomethacin is illustrated. The data for collagen in the absence of indomethacin are not included in Fig. 2 because of the uncertainty in determining the maximum $[\text{Ca}^{2+}]_i$ in aggregating platelets as discussed above. Fig. 2 as a whole illustrates that collagen is capable of liberating substantial amounts of free arachidonate at near to resting $[\text{Ca}^{2+}]_i$ levels, whereas ionomycin requires $[\text{Ca}^{2+}]_i$ levels at least an order of magnitude higher (in the μM range). These effects of ionomycin and collagen on fura-2-loaded platelets were found to be identical in unloaded platelets (results not shown). This indicates a substantially different $[\text{Ca}^{2+}]_i$ requirement for the mechanisms utilized by these two agonists for the liberation of arachidonate. From Table 2 the principal source of the $[^3\text{H}]$ arachidonate liberated by collagen appears to be $[^3\text{H}]\text{PtdCho}$, which was depleted by about 20%, although this was reduced to around 10% in indomethacin-treated platelets. In seven different experiments collagen depleted $[^3\text{H}]\text{PtdCho}$ by $17 \pm 4\%$ ($\pm \text{s.d.}$); in indomethacin-treated cells this depletion was $9 \pm 7\%$ ($\pm \text{s.d.}$) in four experiments. The other major phospholipids that were investigated ($[^3\text{H}]\text{PtdSer}$ and $[^3\text{H}]\text{PtdEtn}$), were depleted by 5–10% by collagen, a quantitatively small loss compared with $[^3\text{H}]\text{PtdCho}$ that was not always statistically significant; these labelling losses however, may not reflect the true loss in mass of arachidonate from these phospholipids (Blackwell *et al.*, 1977; Irvine, 1982).

As seen in Table 2, collagen caused a 45% depletion in $[^3\text{H}]\text{PtdIns}$ that was reduced to around 10% depletion by indomethacin. In seven different experiments platelet activation by 10 μg of collagen/ml resulted in a $41 \pm 7\%$ ($\pm \text{s.d.}$) depletion in $[^3\text{H}]\text{PtdIns}$. In four separate experiments 10 μM -indomethacin reduced $[^3\text{H}]\text{PtdIns}$ depletion to $10 \pm 6\%$ ($\pm \text{s.d.}$). These results imply that the bulk of $[^3\text{H}]\text{PtdIns}$ is lost due to the activity of cyclo-oxygenase products such as TxA_2 generated by the platelets and that a minor component of $[^3\text{H}]\text{PtdIns}$ depletion is a consequence of collagen-platelet interaction

Table 2. Platelet lipid changes associated with collagen-induced changes in $[\text{Ca}^{2+}]_i$

Lipids from the platelet samples shown in Fig. 3 were analysed as described in the Experimental section and for Table 1. The results are mean values $\pm \text{s.d.}$ of triplicate determinations from one of four similar experiments.

Additions	$[^3\text{H}]\text{Lipids (c.p.m.}/2 \times 10^8 \text{ cells)}$					
	Arachidonic acid	PtdCho	PtdSer	PtdEtn	PtdIns	PtdA
Controls	2991 ± 660	53269 ± 1923	17796 ± 1305	22637 ± 1041	10101 ± 536	1217 ± 274
Collagen (10 $\mu\text{g}/\text{ml}$)	$11930 \pm 959^{***}$	$41524 \pm 2646^{***}$	16178 ± 812	$19921 \pm 912^*$	$5472 \pm 363^{***}$	$2753 \pm 203^{**}$
Collagen (10 $\mu\text{g}/\text{ml}$) + indomethacin (10 μM)	$11328 \pm 3089^{**}$	$46080 \pm 2414^*$	17333 ± 1185	20949 ± 1628	9182 ± 320	1475 ± 434

Table 3. Agonist-induced stimulation of $[Ca^{2+}]_i$, phosphoinositide turnover and liberation of arachidonic acid

In the presence of 1 mM- $[Ca^{2+}]_o$, 0.8 ml aliquots of fura-2-loaded, $[^3H]$ arachidonate- and $[^{32}P]P_i$ -labelled platelets were stimulated for 3 min at 37 °C, as indicated. Maximum $[Ca^{2+}]_i$ levels were determined from fura-2 fluorescence and radioactive lipids were analysed as described for Table 1 and in the Experimental section. The results shown are mean values $\pm$ S.D. of triplicate determinations from one of three similar experiments.

Additions	Maximum $[Ca^{2+}]_i$ (nM)	Lipids (c.p.m./ 2×10^8 cells)		
		$[^3H]PtdA$	$[^{32}P]PtdA$	$[^3H]$ Arachidonic acid
Control	110 $\pm$ 10	1074 $\pm$ 252	570 $\pm$ 209	6432 $\pm$ 93
Collagen (10 μ g/ml)	385 $\pm$ 44	1891 $\pm$ 309*	2406 $\pm$ 130***	12719 $\pm$ 1005***
Collagen (10 μ g/ml) + indomethacin (10 μ M)	97 $\pm$ 2	1287 $\pm$ 135	1456 $\pm$ 149**	12269 $\pm$ 129***
Ionomycin (30 nM)	755 $\pm$ 36	701 $\pm$ 292	530 $\pm$ 133	5732 $\pm$ 490

per se. The contribution of the depletion in $[^3H]PtdIns$ to the liberation of $[^3H]$ arachidonate in response to collagen is difficult to interpret, since both direct deacylation and stimulation of the phosphoinositide cycle could result in $[arachidonoyl-^3H]PtdIns$ depletion. These effects of collagen (in the presence of indomethacin) on $[^3H]PtdCho$ and $[^3H]PtdIns$ levels in fura-2-loaded platelets were found to be no different from the effects of collagen on unloaded platelets.

Stimulation of phosphoinositide turnover may be readily assessed by measuring the formation of the PtdA derived from diacylglycerol formed as a consequence of PLC hydrolysis of the phosphoinositides. In Table 2 collagen caused a 2-fold elevation of $[^3H]PtdA$ levels that was reduced to statistical insignificance by indomethacin. Using $[^{32}P]P_i$ as a label, i.e. measurement of $[^{32}P]PtdA$ formation, provides a more sensitive index of stimulated phosphoinositide turnover than the formation of $[arachidonoyl-^3H]PtdA$ (Holmsen *et al.*, 1981). The results from an experiment similar to that depicted in Fig. 3, but using platelets double-labelled with $[^3H]$ arachidonate and $[^{32}P]P_i$, are summarized in Table 3. The $[Ca^{2+}]_i$ and $[^3H]$ arachidonate liberation profiles in response to collagen and ionomycin were as previously discussed. In this particular experiment collagen caused less than 2-fold stimulation of $[^3H]PtdA$ formation but a 4–5-fold stimulation of $[^{32}P]PtdA$ formation. Such an increase in sensitivity in monitoring collagen-induced PtdA formation is further evident in the presence of indomethacin. Under these conditions (Table 3), collagen evoked an elevation in $[^3H]PtdA$ of around 20% over control levels which, due to the scatter in the data, was not statistically significant. However, the corresponding levels of $[^{32}P]PtdA$ were 2.5-fold over the control levels. In three similar experiments collagen in the presence of indomethacin stimulated $[^{32}P]PtdA$ levels to $248 \pm 32\%$ of the control levels ($\pm$ S.D.). Ionomycin at the concentration indicated caused no $[^{32}P]PtdA$ formation. Hence it appears that collagen provokes a stimulation of phosphoinositide turnover that is reinforced by the generation of TxA_2 .

Effect of PMA on the liberation of $[^3H]$ arachidonic acid

The compound PMA can activate platelets in the absence of an elevation in $[Ca^{2+}]_i$ (Rink *et al.*, 1983) an effect of PMA that has been attributed to the activation of protein kinase C. We have investigated whether PMA

Fig. 4. Effect of PMA on the liberation of arachidonic acid

In the presence of 1 mM- $[Ca^{2+}]_i$ and 10 μ M-indomethacin, 0.8 ml samples of fura-2-loaded platelets prepared as described for Fig. 1 were exposed to: (a) dimethyl sulphoxide (controls), (b) 30 nM-ionomycin, (c) 20 nM-PMA, (d) 30 nM-ionomycin plus 20 nM-PMA, and (e) collagen (10 μ g/ml). At 150 s after agonist additions the reactions were quenched and $[^3H]$ arachidonate levels were analysed as described in the Experimental section. The results shown are mean values $\pm$ S.D. of duplicate or triplicate determinations from one of three similar experiments.

may regulate deacylation of phospholipids either directly or in a synergistic fashion, in concert with $[Ca^{2+}]_i$. In Fig. 4 the effect of 20 nM-PMA alone, and in the presence of 30 nM-ionomycin, on $[^3H]$ arachidonate levels in indomethacin-treated platelets is shown in comparison with that of 10 μ g of collagen/ml. Neither PMA nor ionomycin alone or in combination caused significant stimulation of $[^3H]$ arachidonate liberation, whereas collagen caused a 2.5-fold stimulation. The fluorescence and absorbance traces corresponding to the free

Fig. 5. Effect of PMA on $[\text{Ca}^{2+}]_i$ and platelet aggregation

Representative fluorescence and absorbance traces from fura-2-loaded platelets stimulated as described for Fig. 4 are shown.

arachidonate levels in Fig. 4 are shown in Fig. 5. When ionomycin was present, the $[\text{Ca}^{2+}]_i$ was elevated to around 800 nM, but with PMA alone $[\text{Ca}^{2+}]_i$ remained at or near basal levels. PMA alone caused platelet aggregation that was augmented both in terms of rate and magnitude by ionomycin. Ionomycin alone caused only shape change. We may conclude therefore that, even though in these experiments PMA, acting at least in part through protein kinase C activation, and ionomycin synergize to exert full secondary aggregation, PMA at the concentration used does not appear to regulate deacylation of platelet phospholipids.

DISCUSSION

In the present study we have investigated the role of $[\text{Ca}^{2+}]_i$ in the regulation of arachidonate liberation during human platelet activation by collagen and the Ca^{2+} ionophore, ionomycin. The results presented indicate that these two agonists can evoke the liberation of free arachidonate from membrane phospholipids by mechanisms which have a differential requirement for $[\text{Ca}^{2+}]_i$. The experimental techniques employed have allowed us to monitor simultaneously $[\text{Ca}^{2+}]_i$ and lipid changes in the same intact platelets. In this respect fura-2 has been shown to have advantages over quin2 since fura-2 does not interfere with the $[^3\text{H}]$ arachidonate labelling pattern of platelet phospholipids. The reason for this difference between quin2 and fura-2 is not clear, but an important factor is likely to be the lower intracellular loading concentration of fura-2, which in turn substantially reduces the amount of formaldehyde produced inside the cells from fura-2-AM.

Ionomycin produced an almost instantaneous, concentration-dependent rise in $[\text{Ca}^{2+}]_i$. The value of using ionomycin experimentally is in the titration of the $[\text{Ca}^{2+}]_i$ that can be achieved. By circumventing receptor-operated

mechanisms, any observed response to ionomycin may be attributed to $[\text{Ca}^{2+}]_i$ alone. An elevation in $[\text{Ca}^{2+}]_i$ to several hundred nanomolar by ionomycin was ineffective in causing $[^3\text{H}]$ arachidonate liberation of arachidonate. However, as indicated in Fig. 2, when the $[\text{Ca}^{2+}]_i$ levels are in the micromolar range, ionomycin provokes Ca^{2+} -dependent liberation of arachidonate. These results are essentially in agreement with those of Rittenhouse & Horne (1984) using quin2-loaded platelets, although these workers did not directly correlate the change in $[\text{Ca}^{2+}]_i$ produced by ionomycin to free arachidonate liberated. The major source of this $[^3\text{H}]$ arachidonate would appear to be $[^3\text{H}]\text{PtdCho}$, and thus we speculate that the Ca^{2+} -dependent liberation of arachidonate observed with ionomycin may reflect Ca^{2+} -calmodulin dependent activation of PLA_2 . Such a possibility is supported by previous reports of PLA_2 activity in platelets activated by the Ca^{2+} ionophore A23187 (Lapetina *et al.*, 1981). A low concentration of ionomycin failed to stimulate phosphoinositide turnover via PLC activation (indicated by $[^{32}\text{P}]\text{PtdA}$ formation) as seen in Table 3. This effect is consistent with the concept that PLC activation in platelets is dependent upon occupation of receptors (Rittenhouse, 1984). In contrast with the immediate elevation in $[\text{Ca}^{2+}]_i$ produced by ionomycin, the elevation in $[\text{Ca}^{2+}]_i$ produced by collagen was preceded by a 10–20 s lag phase. Such an observation in fura-2-loaded platelets is consistent with the pattern of $[\text{Ca}^{2+}]_i$ changes produced by collagen in quin2-loaded platelets (Rink & Hallam, 1984). From the studies with indomethacin shown in Fig. 3, the elevation in $[\text{Ca}^{2+}]_i$ in response to collagen appears to be almost totally dependent on cyclo-oxygenase products. Prostaglandin endoperoxides and TxA_2 are known to interact with TxA_2 receptors on the platelet plasma membrane that are coupled to an elevation in $[\text{Ca}^{2+}]_i$ (Pollock *et al.*, 1984).

The results summarized in Fig. 2 indicate that collagen initiates the liberation of free arachidonate both in the absence and in the presence of indomethacin at $[\text{Ca}^{2+}]_i$ levels at which ionomycin-induced elevation of $[\text{Ca}^{2+}]_i$ is ineffective. Therefore, collagen appears to use mechanisms additional to or independent of $[\text{Ca}^{2+}]_i$. In the absence of indomethacin, the 20% depletion in PtdCho caused by collagen (Table 2) can more than account for the complementary increase in $[^3\text{H}]$ arachidonate, suggesting that deacylation of this phospholipid is the principal source of free arachidonate during platelet activation. However, we should emphasize that these remarks apply only to the radiolabelled acid; previous studies on platelets have shown clear differences between mass and radiolabelling data (Blackwell *et al.*, 1977; Irvine, 1982), and probably deacylation of PtdEtn is occurring also in the present experiments. Two possible sources of loss in $[^3\text{H}]$ arachidonate radioactivity may contribute to the discrepancy between radioactivity gained in $[^3\text{H}]$ arachidonate and lost from $[^3\text{H}]$ phospholipids observed in Table 2. These are (i) bioconversion of $[^3\text{H}]$ arachidonate to more hydrophilic eicosanoids that are lost to the aqueous phase of the lipid extraction, and (ii) transacylation of $[^3\text{H}]$ arachidonate to other lipid classes (Irvine & Dawson, 1979; Kramer & Deykin, 1983).

To what extent collagen-platelet interaction *per se* contributes to the effects on platelet lipids, and to what extent these effects are dependent upon the generation of prostaglandin endoperoxides and TxA_2 , can be assessed

from the results obtained in collagen in the presence of indomethacin. Although the depletion in [^3H]PtdCho is attenuated in the presence of indomethacin, the most marked effect of indomethacin is on [^3H]PtdIns depletion and [^3H]PtdA accumulation. This sensitivity of collagen-induced changes in PtdIns to indomethacin (75% inhibition) is in agreement with previous reports (Rittenhouse-Simmons & Allen, 1982; Sano *et al.*, 1983). Although [^3H]PtdA accumulation is reduced to a statistically insignificant level, stimulation of [^{32}P]PtdA formation in the presence of indomethacin (Table 3) indicates that collagen itself may activate PLC. These results are in agreement with Sano *et al.* (1983), but contradictory to the results of Siess *et al.* (1983) who found no stimulation of [^{32}P]PtdA formation by collagen in the presence of indomethacin. However the recent report of InsP_3 accumulation in indomethacin-treated platelets stimulated with collagen (Watson *et al.*, 1985) supports the probability that collagen-platelet interaction directly activates PLC.

Previous studies have considered direct deacylation of phospholipids to be a consequence of Ca^{2+} -calmodulin-dependent activation of PLA_2 (Pickett *et al.*, 1977; Wong & Cheung, 1978). However collagen fails to elevate [Ca^{2+}] $_i$ sufficiently to activate the enzyme by this means. It is known that oleoyl acetyl glycerol can shift the Ca^{2+} -dependency curve for platelet dense granule secretion to the left (Knight & Scrutton, 1984). Recently evidence from neutrophils (Volpi *et al.*, 1985) has shown that PMA, presumably by mimicking endogenously-produced diacylglycerol to activate protein kinase C, acts synergistically with ionomycin to stimulate arachidonate liberation. The negative results obtained with PMA in the presence of ionomycin (Fig. 4) would suggest that such a mechanism for the activation of PLA_2 may not exist in human platelets. Mobley & Tai (1985) have observed synergism between ionomycin and PMA for TxB_2 formation, whilst Halenda *et al.* (1985) have demonstrated an enhancement, by PMA, of arachidonate liberation by A23187. However, both these groups found that high concentrations of PMA and/or calcium ionophores were required. Their data suggest that the concentration-response curve for PMA in the presence of ionophore, or vice versa, with respect to liberating arachidonic acid is to the right of the concentration response curves in inducing platelet aggregation.

Thus the results of Mobley & Tai (1985) and Halenda *et al.* (1985) are not inconsistent with ours, and our data (Fig. 4) emphasize that the ability of collagen to induce diacylglycerol production, and therefore activate protein kinase C, does not explain its efficacy in stimulating phospholipid deacylation. A more plausible mechanism for regulating arachidonate liberation in response to collagen at low [Ca^{2+}] $_i$ is membrane perturbation (Dawson, 1982; Irvine, 1982; Dawson *et al.*, 1984). Stress-induced deacylation may result from macromolecular collagen fibrils binding to several sites on the platelet plasma membrane causing lateral strain across the membrane surface. In mast cells, liberation of arachidonate by compound 48/80 may also involve membrane perturbation activation of PLA_2 (Okano *et al.*, 1985).

In the absence of indomethacin, the role of arachidonate metabolites (prostaglandin endoperoxides and TxA_2) in platelet responses to collagen may be simplistically assessed as a mechanism to reinforce the effects of

collagen. The thromboxane-dependent elevation in [Ca^{2+}] $_i$ and the stimulation of phosphoinositide turnover as a consequence of TxA_2 receptor occupancy may have a more important role to play in the regulation of the biochemical pathways concerned with platelet shape, change and aggregation rather than with arachidonate liberation *per se* (compare the synergism between PMA and ionomycin for platelet aggregation but not with arachidonate liberation). It has been suggested that metabolites of arachidonate exhibit positive co-operativity with collagen to regulate the liberation of arachidonate itself (Rittenhouse-Simmons & Allen, 1982). Hence, although collagen-platelet adhesion causes the initial deacylation, subsequent synthesis of eicosanoids may further contribute to this process. The thromboxane dependent elevation in [Ca^{2+}] $_i$ also indicates a fundamental difference between TxA_2 receptors and collagen binding sites on platelets. Whereas the former are coupled to stimulation of Ca^{2+} influx, the latter would not appear to be so.

In conclusion, we have demonstrated that collagen can induce the liberation of free arachidonate at, or near, resting [Ca^{2+}] $_i$, and the mechanism of this activation remains to be investigated.

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