Biochem. J. (1984) **218**, 819–827
Printed in Great Britain

Stimulus–response coupling in human platelets

Changes evoked by platelet-activating factor in cytoplasmic free calcium monitored with the fluorescent calcium indicator quin2

Trevor J. HALLAM, Ana SANCHEZ and Timothy J. RINK
Physiological Laboratory, Downing Street, Cambridge CB2 3EG, U.K.

(*Received 12 September 1983/Accepted 22 November 1983*)

The role of changes in cytoplasmic free calcium, $[Ca^{2+}]_i$, in the responses to platelet-activating factor (PAF) was studied in human platelets loaded with the fluorescent calcium indicator, quin2. In the presence of 1 mM external calcium, PAF raised $[Ca^{2+}]_i$ 8–10-fold in a few seconds to peak near 1 μM. $[Ca^{2+}]_i$ then declined over several minutes towards the basal level. In the absence of external calcium there was a much smaller increase in $[Ca^{2+}]_i$ of similar pattern. These findings suggest that PAF increases $[Ca^{2+}]_i$ partly by discharge of internal Ca^{2+}, but mainly by stimulated influx. Blockade of cyclo-oxygenase with aspirin only slightly reduced the $[Ca^{2+}]_i$ changes, indicating that thromboxane A_2 is not a major mediator of the calcium movements. In control conditions PAF could stimulate shape-change, aggregation and secretion. Aggregation and secretion were roughly halved by blockade of cyclo-oxygenase. Shape-change and secretion still occurred under conditions where the $[Ca^{2+}]_i$ rise was small or suppressed, indicating a role for intracellular activators other than Ca^{2+}. The possible involvement of products of phosphoinositide breakdown is discussed.

Platelet-activating factor (PAF), 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine, is produced by and released from various types of cell that mediate inflammatory and allergic responses and may be responsible for several features of anaphylactic shock (Demopoulos *et al.*, 1979). PAF is also a powerful activator of blood platelets, active *in vitro* in the nanomolar range, though its role in normal and abnormal haemostatis is as yet largely unknown. PAF stimulates rapid shape-change, secretion of the contents of both dense granules and alpha granules and aggregation. Since PAF can be formed in stimulated platelets it has been proposed that PAF may partly mediate the effects of other agonists. However, at most a minor role for endogenous PAF in the responses of human platelets to other agonists is indicated by desensitization studies (Hallam *et al.*, 1983*a*), the presence of normal responses in subjects deficient in an enzyme required for the formation of PAF (Sturk *et al.*, 1983) and observations reported here.

Our main aim has been to investigate the processes of stimulus–activity coupling and in particular the role of cytoplasmic free Ca^{2+}, $[Ca^{2+}]_i$, in the response to PAF. $[Ca^{2+}]_i$ has been directly measured in intact human platelets from the fluorescence of the intracellularly-trapped indicator quin2 (Tsien *et al.*, 1982). Changes in $[Ca^{2+}]_i$ evoked by PAF have been examined together with their dependence on external calcium. Shape-change, secretion and aggregation were monitored in parallel in the same cell preparations, so that $[Ca^{2+}]_i$ changes could be compared with the cellular responses. The effects of PAF could also be observed when conditions were manipulated to suppress $[Ca^{2+}]_i$ changes. Considering this data, along with previous observations of the $[Ca^{2+}]_i$ levels required to initiate these responses when $[Ca^{2+}]_i$ is elevated by a calcium ionophore (Rink *et al.*, 1982), one can assess how far calcium mediates the effects of PAF and how far other intracellular activators may also be involved. PAF is known to stimulate formation of thromboxane A_2 and there has been considerable discussion as to its role in mediating the effects of PAF in human platelets (Marcus *et al.*, 1981; McManus *et al.*, 1981; Chesney *et al.*, 1982). We have therefore also examined the effects of inhibiting conversion of

Abbreviation used: PAF, platelet-activating factor.

arachidonate to prostaglandin endoperoxides and thromboxane A_2. Part of this work has been previously reported in abstract form (Hallam *et al.*, 1983*b*; Rink, 1983).

Experimental

Preparation of cells

Blood was freshly drawn from healthy volunteers who had denied taking aspirin for the previous 10 days and mixed with one-sixth volume of acid citrate/dextrose prepared as described by Aster & Jandl (1964), to give a final whole blood citrate concentration of 22mM and a pH of 6.5. Platelet-rich plasma was prepared by centrifugation for 5min at 700*g* at room temperature. The platelets were loaded with quin2 by incubating the platelet-rich plasma at 37°C for 30min with 15 μM-quin2 acetoxymethyl ester (Tsien *et al.*, 1982; Rink *et al.*, 1982). In some experiments 10nM-prostaglandin I_2 (prostacyclin) was also included to prevent activation (Moncada *et al.*, 1982). The platelets were pelleted by centrifugation at 500*g* at room temperature for 20min. In practice it was not clear that prostaglandin I_2 treatment had any beneficial effect. Once the supernatant had been discarded, the cells were gently resuspended in physiological saline of the following composition: 145mM-NaCl, 5mM-KCl, 1mM-$MgSO_4$, 10mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 10mM-glucose, pH7.4 at 37°C. The cell suspensions were restored to a density of approx. 1×10^8 cells·ml^{-1} and usually incubated at 37°C for 15–20min. They were then left at room temperature (20–25°C) in a plastic tube. Prior to experimental procedures the external calcium concentration was adjusted by addition of $CaCl_2$ or EGTA as required and the cells were equilibrated at 37°C for about 5min.

Measurement of $[Ca^{2+}]_i$

The measurement of $[Ca^{2+}]_i$ from the fluorescence of the intracellularly trapped quin2 was similar to that previously reported (Tsien *et al.*, 1982; Rink *et al.*, 1982). The suspension was placed in a cylindrical cuvette in a specially constructed thermostatted holder, in a Perkin-Elmer MPF 44A spectrophotometer and the fluorescence signal with excitation 339nm, emission 500nm, was recorded on a chart recorder. The cuvette could be continuously stirred using a magnetic stir-bar. The calibration of the fluorescence signal in terms of $[Ca^{2+}]_i$ was based on procedures detailed elsewhere (Tsien *et al.*, 1982). In short, at the end of each procedure one obtains the fluorescence level from calcium-saturated indicator ($F_{max.}$) and from calcium-free medium ($F_{min.}$) by exposing the quin2 to first high, 1mM, and then very low, <1nM, $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$, reported by the observed suspension fluorescence F, is given by:

$$[Ca^{2+}]_i = 115 \cdot \frac{(F - F_{min.})}{(F_{max.} - F)} \text{ nM}$$

$F_{max.}$ can be obtained by (*a*) adding a large concentration of calcium ionophore in the presence of external calcium or (*b*) releasing the indicator into high-calcium medium by lysing the cells with detergent, e.g. Triton X 100, or disrupting the cells with a probe sonicator. To obtain $F_{min.}$ the fluorescence is measured after addition of EGTA in sufficient quantity to be in excess of the calcium concentration followed by adjustment of the pH to approx. 8.5 with Tris base to make the effective K_d for Ca·EGTA $< 10^{-9}$M. An alternative method is to quench the dye fluorescence by addition of 0.1–0.5mM free Mn (Tsien *et al.*, 1982; Hesketh *et al.*, 1983) to give just the autofluorescence of the cells, AF. $F_{min.}$ is then calculated from:

$$F_{min.} = AF + \frac{(F_{max.} - AF)}{6}$$

(Rink *et al.*, 1983). The measurement of $F_{max.}$ requires care as contamination of the suspension with trace amounts of transition metals can partially quench the signal. This problem is largely obviated by adding 0.1–0.5mM calcium diethylenetriamine penta-acetate (Tsien *et al.*, 1982), a chelator with a very high affinity for such metals and only a low affinity for calcium ions.

Measurement of shape change, secretion and aggregation

Shape change and aggregation were assessed by a standard turbimetric technique (Born, 1962) at 37°C, stirring at 1000rev./min. Simultaneous measurement of ATP secretion and of the aggregatory response was obtained by addition of 10 μl of luciferin/luciferase reagent (Chronolume, Chronolog) to 490 μl of platelet suspension. The aggregatory and secretory responses were then monitored by absorbance and luminescence using a Chronolog Lumiaggrometer model 400 connected to a two-channel chart recorder.

Materials

PAF was obtained from Calbiochem and was a mixture of 1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine and 1-*O*-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine. For this reason the concentrations of PAF are given in ng/ml rather than nmol/l. Human thrombin was also obtained from Calbiochem. Ionomycin was obtained from Squibb. EGTA was Fluka, puriss grade. The quin2 acetoxymethyl ester was synthesized by Lancaster

Synthesis. Other reagents were Analar grade or the highest purity conveniently available.

Results

PAF-induced changes in $[Ca^{2+}]_i$

In the presence of physiologically normal, 1 mM, external calcium, PAF produced within a few seconds an approx. 10-fold rise in $[Ca^{2+}]_i$ from the basal level near 0.1 μM. The rise was only short-lived and $[Ca^{2+}]_i$ declined towards the resting level within a few minutes. Fig. 1(*a*) shows a typical response to a maximally effective concentration of PAF, 20 ng/ml. Similar responses were obtained with 2 ng of PAF/ml. The mean peak $[Ca^{2+}]_i$ with 20 ng of PAF/ml was 886 $\pm$ 64 nM (S.E.M.; $n = 28$). A second application of PAF was almost without effect (Fig. 1*a*) possibly reflecting rapid receptor desensitization (Henson, 1976). Subsequent application of the calcium ionophore, ionomycin, could rapidly elevate $[Ca^{2+}]_i$ to the maximum discernible value, as in Fig. 1(*a*). Furthermore, after the decline of the response to PAF the cells could still show a large response to thrombin, as shown in Fig. 1(*b*). The rise in $[Ca^{2+}]_i$ was similar to that seen when this concentration of thrombin, 0.5 unit/ml, is applied alone (Rink *et al.*, 1982), being larger and longer sustained than that produced by PAF.

The responses shown in Fig. 1 were obtained from cells which were not continuously stirred. They therefore did not form aggregates, although shape change and secretion occur under these conditions. Fig. 2 shows simultaneously recorded quin2 fluorescence and absorbance change produced by PAF when the fluorimeter cuvette was continuously stirred. The initial part of the $[Ca^{2+}]_i$ rise is similar to that in unstirred suspension, and the absorbance record indicates rapid shape-change. The fluorescence, however, then starts to decline along with the onset of aggregation. This is due to the aggregates interfering with the fluorescence signal and constitutes a limitation of the technique. In conditions in which stirred cells do not aggregate there is no obvious difference in the quin2 signals from stirred and non-stirred preparations.

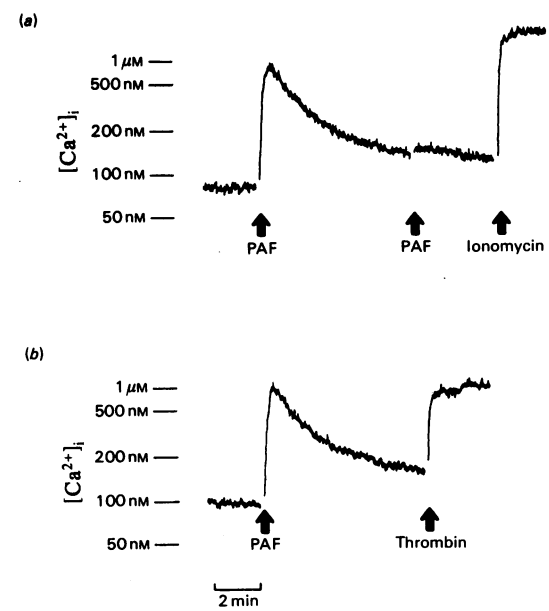

Fig. 1. *Effect of PAF on* $[Ca^{2+}]_i$ *in 1 mM-*Ca^{2+} *medium*
PAF, 20 ng/ml, was added as indicated. The Figure also shows the effect of subsequent addition of ionomycin, 100 nM (*a*), or thrombin, 0.5 unit/ml (*b*). The appropriate calibration scales for $[Ca^{2+}]_i$ are shown at the left-hand side of each fluorescence trace. Gaps in the fluorescence recording show periods for additions.

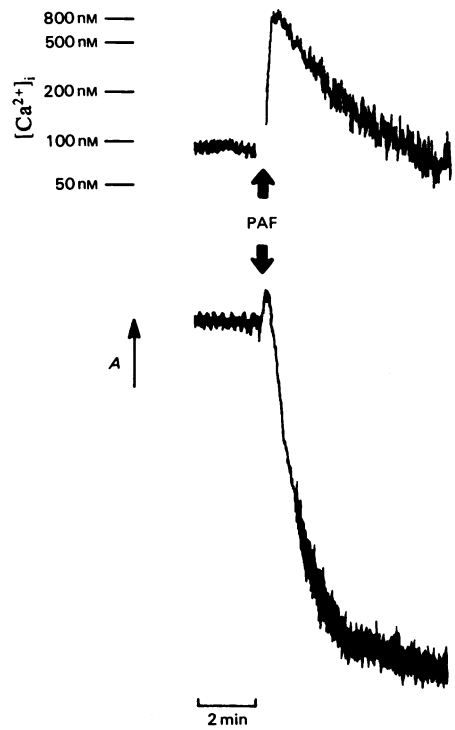

Fig. 2. $[Ca^{2+}]_i$ *and aggregation monitored in same cuvette*
PAF, 20 ng/ml, was added to an aliquot of platelet suspension in the presence of 1 mM external Ca^{2+}. The cuvette was continuously stirred. The Figure shows simultaneous recordings of quin2 fluorescence, upper trace, and absorbance, lower trace.

Fig. 3 shows responses to 20ng of PAF/ml in nominally calcium-free medium containing 1.0mM-EGTA. Under these conditions influx of calcium is expected to be negligible and therefore any change in $[Ca^{2+}]_i$ may be attributed to discharge of intracellular calcium into the cytoplasm. PAF at 20ng/ml produced only a small transient rise in $[Ca^{2+}]_i$ to 170nM from the basal level of 50nM. A further addition of the same dose of PAF produced no additional response (Fig. 3*a*). However, a subsequent addition of thrombin produced a larger rise in $[Ca^{2+}]_i$ as expected, since thrombin appears to discharge virtually the same internal calcium pool into the cytoplasm as does ionomycin (Rink *et al.*, 1982). Fig. 3(*b*) shows a similar experiment in which a subsequent addition of a maximally effective dose of ionomycin, 100nM, produced a larger and maintained elevation in $[Ca^{2+}]_i$. These results suggest that PAF is able to mobilize only part of the releasable store of calcium. From the percentage change in quin2 fluorescence and the quin2 content of the cell, one can estimate the amount of Ca^{2+} transferred from the internal store to the quin2 in the cytoplasm (Pozzan *et al.*, 1982). Typically this was between 0.1 and 0.2mmol per litre of cell. The discharge of Ca^{2+} into the cytoplasm would be larger by the amount bound to endogenous calcium buffers.

It can be seen in Fig. 1 that the calibration scale for $[Ca^{2+}]_i$ is not linear and that as $[Ca^{2+}]_i$ approaches 1μM the increases in fluorescence become small. This results from the basic properties of the simple 1:1 Ca^{2+}–quin2 stoichiometry as is fully discussed elsewhere (Tsien *et al.*, 1982). Two effects of this behaviour are that the accuracy of the measurement above micromolar concentrations becomes increasingly poor, and that the raw traces do not give a fair visual impression of the quantitative nature of the $[Ca^{2+}]_i$ response. In particular the difference between the response in 1mM-calcium and calcium-free medium are underemphasized. Fig. 4 demonstrates this by showing deconvoluted plots of the responses to PAF from Fig. 1(*a*) and Fig. 3(*a*).

There has been considerable discussion over the extent to which thromboxane A_2 mediates the effects of PAF (e.g. Marcus *et al.*, 1981; Chesney *et al.*, 1982), and also speculation that thromboxane A_2 may play an important role in calcium mobilization (Gerrard *et al.*, 1981). We have

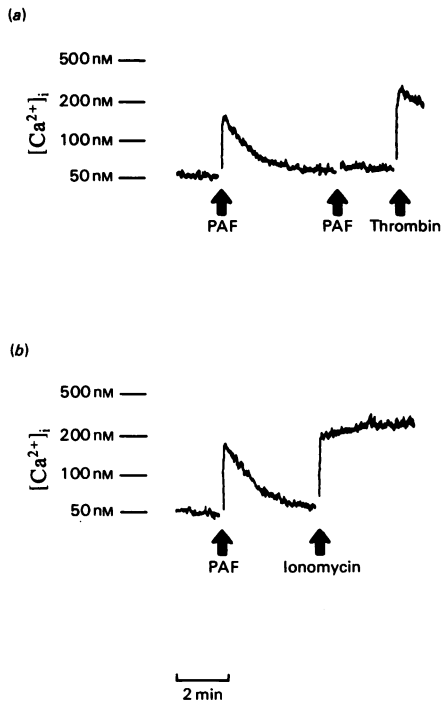

Fig. 3. *Effect of PAF on $[Ca^{2+}]_i$ in the absence of external Ca^{2+}*
The quin2 fluorescence traces show the effect of addition of PAF, 20ng/ml, followed by: a further addition of PAF, 20ng/ml, and then 0.5 units of thrombin/ml in (*a*); or 100nM-ionomycin in (*b*). Prior to stimulation the cells were incubated with 1mM-EGTA for several minutes.

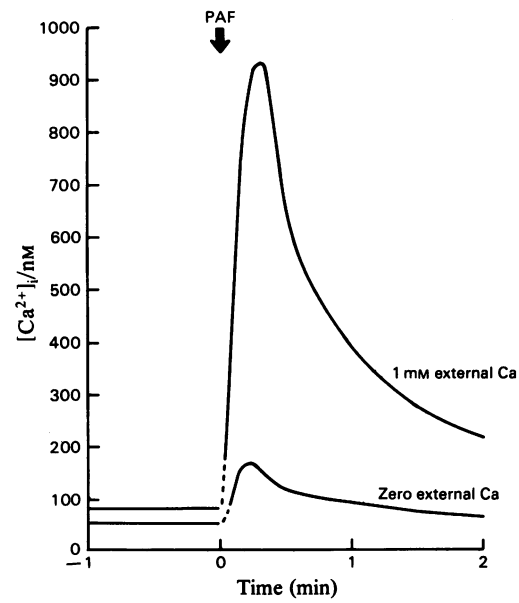

Fig. 4. *$[Ca^{2+}]_i$ responses redrawn on a linear scale*
The Figure shows deconvoluted plots of the rise in $[Ca^{2+}]_i$ on stimulation with 20ng of PAF/ml which occur in the presence or absence of external Ca^{2+}. The original traces from which the linear $[Ca^{2+}]_i$ plots were taken are shown in Figs. 1(*a*) and 3(*a*). The deconvolution was done by hand.

recently reported that arachidonate and stable analogues of thromboxane A_2 can increase $[Ca^{2+}]_i$, mainly by stimulated calcium influx (Hallam *et al.*, 1983*c*). PAF-induced changes in $[Ca^{2+}]_i$ were therefore examined before and after formation of thromboxane A_2 from arachidonate was prevented by blockade of cyclo-oxygenease. Fig. 5 shows typical results. In Figs. 5(*a*), and 5(*b*) it can be seen that aspirin only slightly reduced the peak $[Ca^{2+}]_i$ produced by 20ng of PAF/ml.

Figs. 5(*c*), and 5(*d*) show that blockage of cyclo-oxygenase produced no detectable reduction in the $[Ca^{2+}]_i$ transient in calcium-free medium. In six such trials the mean peak $[Ca^{2+}]_i$ was 161 ± 8nM in the controls and $152 + 15$nM in the inhibited cells. These findings suggest that thromboxane A_2 is not involved in the discharge of internal Ca^{2+}, but may contribute part of the stimulated calcium-influx. PAF is evidently able to promote a substantial increase in $[Ca^{2+}]_i$ without any requirement for thromboxane production.

Shape-change, secretion, aggregation

Fig. 6 shows simultaneous recordings of shape-change, aggregation and secretion of ATP run in parallel in the same batch of quin2-loaded platelets shown in Fig. 5. Fig. 6(*a*) shows that in the presence of 1mM-calcium, 20ng of PAF/ml gave shape change, aggregation and substantial, brisk secretion of ATP, showing that cell function was apparently not adversely affected by quin2.

In calcium-free medium, Fig. 6(*c*), there was still shape-change and secretion of ATP in response to PAF, though the latter was less than that seen in the presence of extracellular calcium (cf. Fig. 6*a*). Aggregates did not form under these conditions, possibly owing to an external requirement for Ca^{2+} in cell–cell interaction. The extent of the ATP secretion in calcium-free medium was variable, usually about 15–20% of that releasable by thrombin under optimal conditions. It should be emphasized that the shape change and secretion were stimulated even though $[Ca^{2+}]_i$ peaked at less than 200nM (cf. Fig. 5*b*).

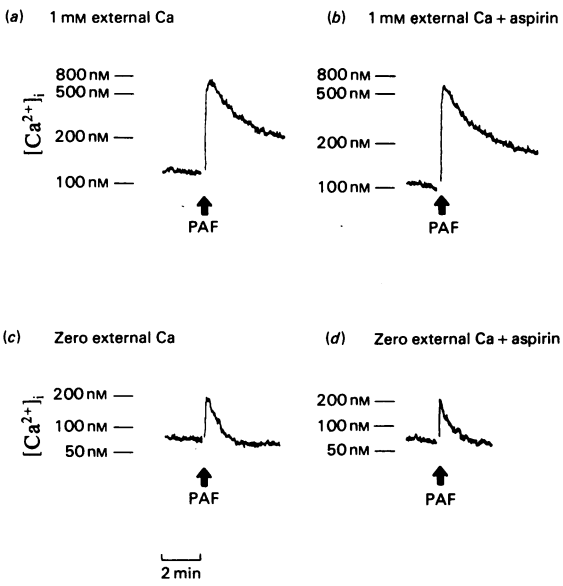

Fig. 5. *Effect of cyclo-oxygenase blockade on the rise in $[Ca^{2+}]_i$ following stimulation with PAF*
The Figure shows the effect of PAF, 20ng/ml, in 1mM-Ca^{2+} medium in the absence (*a*) or presence (*b*) of 100μM-aspirin, and also in the absence of external Ca^{2+}, in the absence (*c*) or presence (*d*) of 100μM-aspirin. These concentrations of aspirin were sufficient to completely block responses to 5μM-arachidonate.

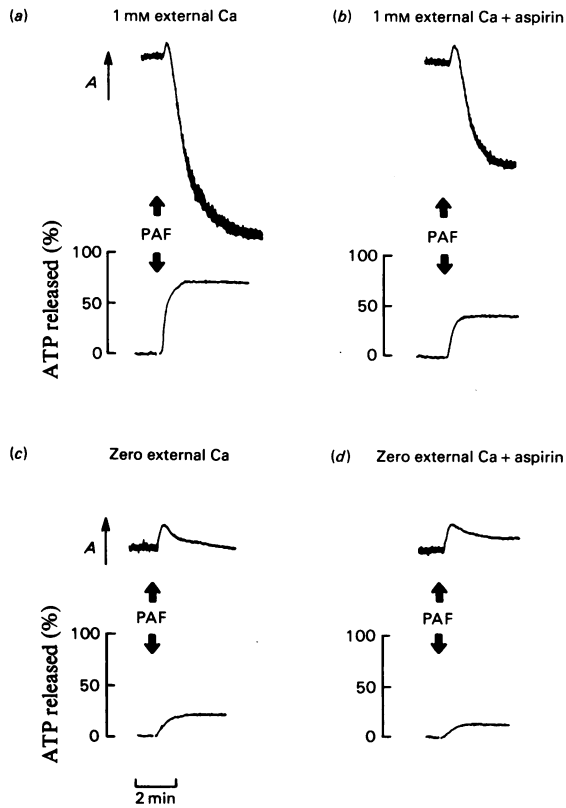

Fig. 6. *Effect of external Ca^{2+} and cyclo-oxygenase inhibition on the aggregatory and secretory responses to PAF in quin2-loaded human platelets*
The Figure shows the simultaneously recorded changes in absorbance and ATP secretion following stimulation with 20ng of PAF/ml. (*a*) 1mM external Ca^{2+}; (*b*) 1mM external Ca^{2+}, 100μM-aspirin; (*c*) 1mM-EGTA; (*d*) 1mM-EGTA, 100μM-aspirin.

Although inhibition of thromboxane A_2 formation had only modest effects on the changes in $[Ca^{2+}]_i$, blockade of cyclo-oxygenase more markedly reduced secretion and aggregation, Figs. 6(*b*) and 6(*d*) show the responses of the same cells shown in Figs. 6(*a*) and 6(*c*), after treatment with 100 μM-aspirin. In this experiment the secretion and aggregation in the presence of 1 mM-calcium were roughly halved. In ten such trials the ATP secretion was reduced from $58 \pm 6\%$ to $16 \pm 4\%$. In the absence of external calcium, the already modest secretion was further reduced, as seen in Fig. 6(*d*), or in some cases completely inhibited. Shape change was not inhibited. As with the stimulated influx therefore, thromboxane A_2 formation may not be essential for cellular responses to PAF, under our experimental conditions, but when thromboxane A_2 formation is permitted, it does add to the stimulation caused by PAF itself.

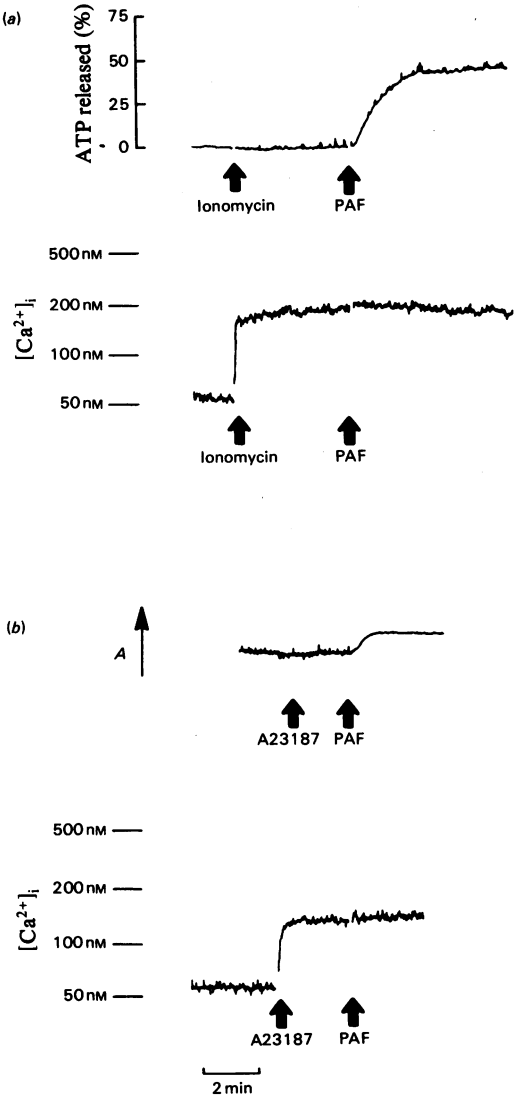

Fig. 7. *Effect of PAF under conditions where a rise in* $[Ca^{2+}]_i$ *is suppressed*

(*a*) Shows parallel recordings of ATP secretion and quin2 fluorescence. The cells were incubated in Ca^{2+}-free medium, with 1 mM-EGTA. Ionomycin, 100 nM, and PAF, 20 ng/ml, were added as indicated. (*b*) Shows parallel recordings of absorbance and quin2 fluorescence of platelets suspended in Ca^{2+}-free conditions and in the presence of 10 μM-indomethacin, A23187, 100 nM, and PAF, 20 ng/ml, were added as indicated. The suspensions were continuously stirred.

It is clear from these experiments that PAF can induce shape-change and secretion at $[Ca^{2+}]_i$ less than 200 nM. This is well below the previously reported $[Ca^{2+}]_i$ thresholds of 0.4 μM for shape-change, and 0.8 μM for secretion, seen when calcium ionophore is used to elevate $[Ca^{2+}]_i$ in these preparations (Rink *et al.*, 1982). Fig. 7(*a*) shows experimental conditions where PAF could stimulate secretion without causing any significant elevation of $[Ca^{2+}]_i$. The cells were suspended in calcium-free medium, and ionomycin, 100 nM, was added to discharge the internally releasable calcium. $[Ca^{2+}]_i$ rose to 200 nM. Parallel records from the Lumiaggrometer indicated that this treatment was insufficient to produce secretion. Subsequent application of 20 ng of PAF/ml produced no further rise in $[Ca^{2+}]_i$ but did evoke approx. 40% secretion of ATP. A similar experiment is shown in Fig. 7(*b*) where PAF evoked shape-change without altering $[Ca^{2+}]_i$. After the internally releasable calcium had been mobilized by the addition of ionophore A23187, $[Ca^{2+}]_i$ rose to approx. 150 nM, insufficient alone to cause shape-change. However, subsequent addition of 20 ng of PAF/ml produced shape-change without any further increase in $[Ca^{2+}]_i$. PAF can apparently stimulate platelets by means other than by elevating $[Ca^{2+}]_i$ as has already been proposed for thrombin (Rink *et al.*, 1982).

Discussion

Ca^{2+} movements

These results show that PAF produces a rapid, transient elevation of $[Ca^{2+}]_i$ in quin2-loaded human platelets. Much of this elevation appears to be due to a stimulated calcium influx since it is much reduced in the absence of external calcium. Previous findings that PAF promotes $^{45}Ca^{2+}$ uptake (Lee *et al.*, 1981, 1983) support this idea. The residual response in calcium-free medium most likely reflects the discharge of calcium from some intracellular store within calcium sequestering organelles. The basis for this proposal is (1) the

demonstration of calcium-accumulating subcellular fractions from platelets with properties similar to that of sarcoplasmic reticulum (Kaser-Glanzmann *et al.*, 1979); and (2) the fact that after addition of calcium ionophore in calcium-free medium, PAF can produce little or no further rise in $[Ca^{2+}]_i$ (Fig. 7). Calcium ionophores are expected to translocate calcium from organelles with high $[Ca^{2+}]_i$ into the cytoplasm and hence deplete such stores so that there is nothing left for PAF to discharge (Fig. 7). An alternative hypothesis is that PAF can promote the release of calcium bound to specific binding sites within the cell such as from the head groups of phosphatidylinositol bisphosphate (Hendrickson, 1969; Gerrard *et al.*, 1981). However, it is not obvious why a calcium ionophore should release such bound calcium and occlude the PAF response.

The source of the internal calcium is not known though the dense tubular system is a strong candidate. The amount of calcium discharged, approx. 0.1–0.2 mmol/litre of cells, is far less than that contained in the dense granules (Costa *et al.*, 1977), which is in any case thought to be complexed and non-exchangeable (Lages *et al.*, 1975). The mitochondria are not likely to be involved since uncouplers such as FCCP (carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone) and valinomycin neither discharge the stores nor prevent natural agonists from doing so (Feinstein, 1980; Smith, 1982).

In assessing the relative importance of calcium influx or release of calcium from internal stores, one must consider the effect of loading platelets with quin2 (see also Tsein *et al.*, 1982; Pozzan *et al.*, 1982). Our platelet preparations normally contain about 1 mM-quin2. It is estimated that this approximately doubles the internal calcium-buffering capacity of the cells (Rink *et al.*, 1982). The peak $[Ca^{2+}]_i$ reached following a briefly stimulated influx would be expected to be somewhat reduced by the presence of quin2 (Tsien *et al.*, 1982; Pozzan *et al.*, 1982). The rise in $[Ca^{2+}]_i$ due to the discharge from a finite store, such as release of calcium from the dense tubular system, would be halved, if the presence of quin2 resulted in a doubling of the buffering capacity. It is therefore expected that the PAF-induced increment in $[Ca^{2+}]_i$ in platelets not loaded with quin2 would be somewhat greater than that observed in these experiments, and the contribution from internal release rather more important.

The time course of the $[Ca^{2+}]_i$ transients shown here could reflect a brief opening of calcium channels in the plasma membrane and dense tubular system. This would cause a pulse of calcium to enter the cytoplasm, raising $[Ca^{2+}]_i$ by 8–10-fold. The subsequent decline could be due to the combination of calcium pumped back across the plasma membrane and back into the dense tubular system. From the initial rate at which the quin2 fluorescence declines following the PAF-stimulated increase, one can estimate a minimum value for the rate of Ca^{2+} removed from the cytoplasm as 0.2 mmol/min per litre of cells. This rate of removal is roughly two orders of magnitude larger than the estimated efflux of Ca^{2+} under basal conditions, 0.25 mmol/h per litre of cells (Smith, 1982), and might imply that the calcium transport is activated considerably more than linearly, perhaps as $([Ca^{2+}]_i)^2$ as has been suggested for the Ca^{2+} pump in intact red cells (Lew *et al.*, 1982).

The brevity of the increased calcium fluxes produced by PAF could reflect degradation of the PAF, some form of receptor desensitization, or inactivation of calcium channels. The failure of a second application of PAF to produce a second response favours the latter two possibilities. The ability of thrombin to provide a further response may argue against channel inactivation if thrombin and PAF act via the same channels. On balance the available evidence from these and other studies (e.g. Henson, 1976; Hallam *et al.*, 1983*a*) is in favour of rapid receptor desensitization.

The membrane mechanisms by which PAF, or any other natural agonist, increases calcium fluxes in platelets remains unknown. The available data argue against any role for voltage-dependent calcium channels of the sort found in muscle and many other cell types (MacIntyre & Rink, 1982; Motulsky *et al.*, 1983). The ability of verapamil to inhibit platelet responses to PAF, and to inhibit the $[Ca^{2+}]_i$ changes (Shaw *et al.*, 1983; T. J. Hallam & T. J. Rink, unpublished work) now appears likely to reflect interference with PAF binding to its receptor (Tuffin *et al.*, 1983) rather than an action at the Ca^{2+} channel. Stimulated influx could occur via receptor-activated calcium channels analogous with the opening of cation channels at neuromuscular junctions by acetylcholine. Some (Michell, 1982) would argue a role for phosphoinositide breakdown, which is a rapid response of human platelets to PAF (MacIntyre & Pollock, 1983; Lapetina & Siegel, 1983). Our data appear to rule out any essential role for thromboxane A_2 in PAF-evoked calcium influx, or internal release (Fig. 5).

The mechanism of triggered internal release remains obscure, just as it does for skeletal muscle and many other tissues. Two possibilities are: (1) some physical consequence of agonist–receptor binding acting through the contiguity of dense tubular system membrane to surface membrane and (2) a chemical messenger. Inositol trisphos-

phate released by phosphoinositide breakdown is one candidate and is formed in thrombin-stimulated cells at the earliest measurable time point so it might be able to act fast enough (Agranoff *et al.*, 1983). Very recently inositol trisphosphate has been shown to release Ca^{2+} from internal organelles of permeabilized pancreatic exocrine cells (Streb *et al.*, 1983) and a direct test of the ability of inositol trisphosphate to discharge Ca^{2+} from the dense tubular system is an obvious next step.

Cell responses

Although stimulated calcium movements are a prominent feature of the response to PAF, the results suggest that other intracellular activators are important in generating the cellular responses. The maximal rise in $[Ca^{2+}]_i$ induced by PAF is normally too small to account for the extent of secretion which ensues when compared with the levels of $[Ca^{2+}]_i$ required for secretion induced by ionophore. PAF could rarely elevate $[Ca^{2+}]_i$ to 1 μM, yet this is the approximate threshold for secretion to be triggered by $[Ca^{2+}]_i$ either when Ca^{2+} fluxes are promoted by calcium ionophore in intact cells (Rink *et al.*, 1982), or when secretion is stimulated by calcium buffers in permeabilized platelets (Knight *et al.*, 1982). Furthermore, PAF can still evoke responses when conditions are manipulated to prevent it increasing $[Ca^{2+}]_i$ (Fig. 7).

Obvious candidates as additional messengers are the immediate breakdown products of phosphatidylinositol bisphosphate. Diacylglycerol activates protein kinase C, which phosphorylates a 40 kDa protein in stimulated platelets (Kaibuchi *et al.*, 1982). This phosphorylation correlates well with secretion (Kaibuchi *et al.*, 1982; Nishizuka, 1983) and exogenous diacylglycerol can stimulate secretion and aggregation without elevating $[Ca^{2+}]_i$ (Rink *et al.*, 1983). Furthermore, modest elevation of $[Ca^{2+}]_i$ with ionophore, too little to directly stimulate secretion, enhances the response to diacylglycerol (Nishizuka, 1983; Rink *et al.*, 1983), so the response to PAF may reflect the combined action of these two messengers.

However there are effects that cannot readily be attributed to this pair of signals. The calcium-independent shape-change like that seen in Fig. 7(*b*) is unlikely to be due to diacylglycerol since neither exogenous diacylglycerol or its pharmacological mimic, phorbol ester (Castagna *et al.*, 1982; Rink *et al.*, 1983), promote this response. A signal is also required to trigger intracellular calcium discharge. Inositol trisphosphate might perform these roles, as has been discussed above for calcium discharge, or there may be yet other excitatory activators to be discovered.

The role of thromboxane A_2

We regard thromboxane A_2 as an endogenously produced agonist which reinforces the response to initially added exogenous agonists. Arachidonate release may be promoted by calcium-activation of phospholipase A_2 or by action of lipases on the diacylglycerol formed as a result of phosphoinositide breakdown. Both of these pathways are clearly available to PAF. There is as yet no demonstrated intracellular target for thromboxane A_2. Rather, it appears to act at surface receptors and cause elevation of $[Ca^{2+}]_i$ (Hallam *et al.*, 1983*c*; Shaw *et al.*, 1983) and breakdown of phosphoinositide (MacIntyre & Pollock, 1982; Siess *et al.*, 1983), thus adding to the supply of intracellular excitatory signals.

We thank the SERC for funding and Ms. Nuala O'Connor for skilled assistance. A.S. held an EMBO Longterm Fellowship.

References

Agranoff, B. W., Murthy, P. & Seguin, E. B. (1983) *J. Biol. Chem.* **238**, 2076–2078

Aster, R. H. & Jandl, J. H. (1964) *J. Clin. Invest.* **43**, 843–855

Billah, M. M. & Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 12705–12708

Born, G. V. R. (1962) *Nature (London)* **194**, 927–929

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, V. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851

Chesney, C. M., Pifer, D. D., Byers, L. W. & Muirhead, E. E. (1982) *Blood* **59**, 582–585

Costa, J. L., Detwiler, T. C., Feinman, R. D., Murphy, D. L., Patlak, C. S. & Pettigrew, K. D. (1977) *J. Physiol. (London)* **264**, 297–306

Demopoulos, C. A., Pinchard, R. N. & Hanahan, D. J. (1979) *J. Biol. Chem.* **254**, 9355–9358

Feinstein, M. B. (1980) *Biochem. Biophys. Res. Commun.* **93**, 593–600

Gerrard, J. M., Peterson, D. A. & White, J. G. (1981) in *Platelets in Biology and Pathology 2* (Gordon, J., ed.), pp. 407–436, Elsevier/North-Holland, Amsterdam

Hallam, T. J., Scrutton, M. C. & Wallis, R. B. (1983*a*) *FEBS Lett.* **162**, 142–146

Hallam, T. J., Rink, T. J. & Sanchez, A. (1983*b*) *Br. J. Pharmacol.* **79**, 351P

Hallam, T. J., Rink, T. J. & Sanchez, A. (1983*c*) *J. Physiol. (London)* **343**, 97P–98P

Hendrickson, H. S. (1969) *Ann. N.Y. Acad. Sci.* **165**, 668

Henson, P. M. (1976) *J. Exp. Med.* **146**, 937–952

Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. & Metcalfe, J.c. (1983) *J. Biol. Chem.* **258**, 4876–4882

Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. & Nishizuka, Y. (1982) *Cell Calcium* **3**, 323–335

Kaser-Glanzmann, R., Gerber, E. & Luscher, E. (1979) *Biochim. Biophys. Acta* **558**, 344–347

Knight, D. E., Hallam, T. J. & Scrutton, M. C. (1982) *Nature (London)* **96**, 256–257

Lages, B., Scrutton, M. C., Holmsen, H., Day, H. J. & Weiss, H. J. (1975) *Blood* **46**, 119–130

Lapetina, E. G. & Siegel, F. L. (1983) *J. Biol. Chem.* **258**, 7241–7244
Lee, T., Malone, B., Blank, M. L. & Snyder, F. (1981) *Biochem. Biophys. Res. Commun.* **102**, 1262–1268
Lee, T., Malone, B. & Snyder, F. (1983) *Arch. Biochem. Biophys.* **223**, 33–39
Lew, V. L., Tsien, R. Y., Miner, C. & Bookchin, R. M. (1982) *Nature (London)* **298**, 478–481
MacIntyre, D. E. & Pollock, W. K. (1982) *Br. J. Pharmacol.* **77**, 466p
MacIntyre, D. E. & Pollock, W. K. (1983) *Biochem. J.* **212**, 433–437
MacIntyre, D. E. & Rink, T. J. (1982) *Thromb. Haemostasis* **47**, 22–26
Marcus, A. J., Safier, L. B., Ullman, H. L., Wong, K. T. H., Broekman, M. J., Weksler, B. B. & Kaplan, K. L. (1981) *Blood* **58**, 1027–1032
McManus, L. M., Hanahan, D. J. & Pinckard, R. N. (1981) *J. Clin. Invest.* **67**, 903–906
Michell, R. H. (1982) *Cell Calcium* **3**, 285–294
Moncada, S., Radomski, M. & Vargas, J. R. (1982) *Br. J. Pharmacol.* **75**, 165P
Motulsky, H. J., Snavely, M. D., Hughes, P. J. & Insel, P. A. (1983) *Circ. Res.* **52**, 226–231
Nishizuka, Y. (1983) *Philos. Trans. R. Soc. London Ser. B* **302**, 101–112
Pozzan, T., Arslan, P., Tsien, R. Y. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 335–340
Rink, T. J. (1983) *Thromb. Haemostasis* **50**, 92
Rink, T. J., Smith, S. W. & Tsien, R. Y. (1982) *FEBS Lett.* **148**, 21–26
Rink, T. J., Sanchez, A. & Hallam, T. J. (1983) *Nature (London)* **305**, 317–319
Shaw, A. M., Brydon, L. J., Pollock, W. K. & MacIntyre, D. E. (1983) *Thromb. Haemostasis.* **50**, 45
Siess, W., Cuatrecasas, P. & Lapetina, E. G. (1983) *J. Biol. Chem.* **258**, 4683–4686
Smith, S. W. (1982) M.Sc. Thesis, University of Cambridge
Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
Sturk, A., Schaap, M. C. L., Asyee, G. M., Schutgens, R. B. H., Bosch, H. V. D., Heymans, H. S. A. & Tencate, J. W. (1983) *Thromb. Haemostasis* **50**, 14
Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
Tuffin, D. P., Wade, P. J., Lunt, D. O. & McCullagh, K. G. (1983) *J. Pharmacol.* **14** *Suppl.* **1**, 67