Biochem. J. (1988) 251, 243–249 (Printed in Great Britain)

Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production

Trevor J. HALLAM,*‡ Jeremy D. PEARSON† and Lindsey A. NEEDHAM†§

*Department of Cellular Pharmacology, Smith Kline and French Research Limited, The Frythe, Welwyn, Herts. AL6 9AR, U.K., and †Section of Vascular Biology, MRC Clinical Research Centre, Harrow, Middx. HA1 3UJ, U.K.

Endothelial cells are known to release prostacyclin (PGI_2) in response to agonists, and this has generally been assumed to be caused, at least in part, by activation of a phospholipase A_2 by elevated concentrations of cytoplasmic free calcium ($[Ca^{2+}]_i$). However, it has been shown in the blood platelet that agonists can cause arachidonate release without elevating $[Ca^{2+}]_i$. In the present study, rigorous analysis is made of the $[Ca^{2+}]_i$-dependence of PGI_2 production in the human umbilical-vein endothelial cell. Thrombin caused a rapid increase in $[Ca^{2+}]_i$ from the resting basal value of 0.1 μM to a peak, within 10–15 s, of approx. 2 μM. In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ then declined back to the resting value within 2–3 min. In the presence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ partly decreased to a new steady-state value of approx. 1 μM. The elevated $[Ca^{2+}]_i$ was maintained while the stimulus and the source of extracellular Ca^{2+} were present, suggesting that it was dependent on influx of Ca^{2+} across the plasma membrane. Thrombin stimulated the production of PGI_2 in the presence or in the absence of extracellular Ca^{2+}. However, the production of PGI_2 was more prolonged in the presence of extracellular Ca^{2+}. Total accumulated amounts of 6-oxo-prostaglandin $F_{1\alpha}$ on stimulation with thrombin without extracellular Ca^{2+} were only 65% of those accumulated with extracellular Ca^{2+} present. Cells depleted of extracellular and intracellular sources of Ca^{2+} by incubation with 1 mM extracellular EGTA and exposing them to ionomycin to discharge intracellular stores produced no elevation of $[Ca^{2+}]_i$ on stimulation with thrombin or production of PGI_2. The threshold $[Ca^{2+}]_i$ required to support the production of PGI_2 was measured to be 0.8–1.0 μM by using different doses of ionomycin selectively to increase $[Ca^{2+}]_i$. This relationship between $[Ca^{2+}]_i$ and PGI_2 production was similar to that produced by using different doses of thrombin. Our results show that the major and probably exclusive intracellular stimulus for the production of PGI_2 by the vascular endothelial cell in response to thrombin is the elevation of $[Ca^{2+}]_i$.

INTRODUCTION

Prostacyclin (PGI_2), a labile and potent vasodilator in many vascular beds and an inhibitor of platelet aggregation, is released from endothelial cells in response to a variety of vasoactive stimuli, including histamine, bradykinin, ATP and thrombin [1–6]. The intracellular mechanisms, activated by external ligands binding to endothelial receptors, that lead to the release of PGI_2 are as yet poorly defined.

There is no conclusive evidence that alterations in cellular concentrations of cyclic AMP or cyclic GMP are causally related to PGI_2 synthesis [7–9]. Several lines of evidence suggest indirectly that agonist-induced elevations of intracellular free calcium concentration ($[Ca^{2+}]_i$) cause PGI_2 production.

(1) Ca^{2+} ionophores increase PGI_2 release from cultured endothelial cells [1]. However, it is not clear from such studies whether effective concentrations of ionophores caused excessive, unphysiological, elevations of $[Ca^{2+}]_i$ to produce the response.

(2) Endothelial PGI_2 production is decreased or blocked by the removal of extracellular Ca^{2+} or by the use of membrane-potential-sensitive Ca^{2+}-channel antagonists [7,10,11].

(3) PGI_2 release in response to agonists or Ca^{2+} ionophores is decreased by agents such as TMB-8 or W7 that can inhibit the movement of Ca^{2+} or the effect of elevated $[Ca^{2+}]_i$ [7,10,11]. These agents are, however, not selective: TMB-8 partly inhibited the conversion of arachidonate (but not PGH_2) into PGI_2 [7], and has been shown in the platelet to be more effective at inhibiting responses mediated by the action of protein kinase C than those mediated by elevated $[Ca^{2+}]_i$ [12].

(4) Most recently, elevations in $[Ca^{2+}]_i$ have been measured directly, by using fluorescent Ca^{2+} indicators, in response to bradykinin, histamine, ATP and thrombin [13–17].

In none of the studies to date has it been demonstrated that the values of $[Ca^{2+}]_i$ stimulated by excitatory agonists were sufficient or even necessary to cause PGI_2 production. The most complete data (e.g. [17]) correlate dose-dependent rises in $[Ca^{2+}]_i$ and PGI_2, which are merely consistent with the interpretation of a causal relationship. Although it is usually assumed that phospholipase A_2 activity (the likely cause of arachidonate

Abbreviations used: PG, prostaglandin; PGI_2, prostacyclin (prostaglandin I_2); $[Ca^{2+}]_i$, cytoplasmic free calcium concentration.

‡ To whom correspondence and reprint requests should be addressed.

§ Present address: British Biotechnology Limited, Brook House, Watlington Road, Cowley, Oxford OX4 5LY, U.K.

liberation for PGI_2 synthesis) is stimulated by an elevation in $[Ca^{2+}]_i$, it has not been shown that this is the only mechanism. Indeed, it has been challenged in studies in the fura-2-loaded human blood platelet, where Pollock *et al.* [18,19] showed that, although elevations in $[Ca^{2+}]_i$ alone caused by the Ca^{2+} ionophore ionomycin can stimulate arachidonic acid release, collagen fails to raise $[Ca^{2+}]_i$ significantly, but does cause the release of substantial amounts of arachidonic acid from phosphatidylcholine. Several other platelet responses can also be evoked by agonists that normally raise $[Ca^{2+}]_i$, but under conditions where the rise is prevented (for review see Hallam & Rink [20]).

Very few investigators have measured $[Ca^{2+}]_i$ in cells attached to a substratum. This is likely to be important in the generation of responses, particularly in cell types such as the endothelium, which forms a flattened, polarized and closely apposed cell monolayer with junctional communication. Hamilton & Sims [21] investigated histamine-induced changes in $[Ca^{2+}]_i$ in indo-1-loaded endothelial cells on micro-carrier beads, and Hassid & Oudinet [22] examined vasopressin-stimulated $[Ca^{2+}]_i$ changes in fura-2-loaded arterial smooth-muscle cells on cover-slips, and correlated these with their measurements (in separate experiments) of PGI_2 synthesis. We recently showed that $[Ca^{2+}]_i$ and its elevation by agonists can be measured at short time intervals in adherent confluent monolayers of fura-2-loaded endothelial cells [13,14].

In the present study we have examined agonist-stimulated rises in $[Ca^{2+}]_i$ and in PGI_2 formation in the same cells, and tested whether the two are causally related. We found clear differences between the $[Ca^{2+}]_i$-dependence of agonist-induced arachidonic acid metabolite production in the endothelial cell and in the blood platelet, the only other cell type where rigorous analysis has been made.

METHODS

Endothelial cells were obtained from 20 cm segments of human umbilical-cord veins by filling the vessel lumen with collagenase (0.5 mg/ml), incubating at 37 °C for 10 min and flushing out the loosened sheets of cells [23]. The cells were collected by centrifugation at 100 *g* for 5 min, resuspended in Medium 199 containing 10% (v/v) foetal-calf serum, 10% (w/v) newborn-calf serum, penicillin and streptomycin (each 50 μunits/ml). Cells from each segment of vessel were plated into 25 cm^2 tissue-culture flasks and incubated at 37 °C in air/CO_2 (19:1). Confluent primary cultures, which formed in 3–6 days, were detached from the flasks by brief treatment with 0.05 trypsin and 0.02% EDTA in phophate-buffered saline (Flow Laboratories, cat. no. 16-891-49), and reseeded in complete medium on to 22 mm × 11 mm glass cover-slips at confluent density.

After 24 h, the cell monolayer was loaded with fura-2 [24,25] by incubation with 2 μM of the membrane-permeant penta-acetoxymethyl ester form of fura-2 (fura-2AM) in complete medium for 45 min at 37 °C (fura-2AM was added from a 2 mM stock in dimethyl sulphoxide; the incubation medium thus contained 0.1% dimethyl sulphoxide). During this period the esterified dye was hydrolysed by cytosolic esterases to its Ca^{2+}-sensitive membrane-impermeant form. At this stage the cell monolayer contained 50–100 μM-fura-2. [Cytoplasmic concentrations of free dye were estimated from the relative amount of Ca^{2+}-sensitive fluorescence and autofluorescence from cells in suspension compared with added fura-2 standards of known concentration. We have assumed that, for cells in monolayer on a cover-slip, the relative ratio of Ca^{2+}-sensitive fluorescence to autofluorescence of the cells, after subtraction of the fluorescence owing to the glass cover-slip, will be equivalent to that of cells in suspension.] The cover-slips were removed and placed in a physiological saline consisting of 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgSO_4$, 10 mM-Hepes, 0.5 mM-$CaCl_2$, 10 mM-glucose, pH 7.4 at 37 °C. The cover-slips were then allowed to cool to 25 °C until ready for use (within 60 min). This procedure minimizes the leakage of active dye from the cells.

For each experiment, a cover-slip was placed across the diagonal of a quartz cuvette containing the above physiological saline but with either 1 mM-$CaCl_2$ or 1 mM-EGTA. The cuvette was then placed in a thermostatically controlled holder at 37 °C in a Spex dual-wavelength excitation fluorescence spectrophotometer. Fluorescence of the fura-2 was then recorded at 0.5 s intervals during the experiment with alternating (30 Hz) excitation wavelengths of 340 nm and 380 nm and measuring emitted light at 500 nm. The slit-widths allowed a band-pass of 4.6 nm on the excitation monochromators and 9.2 nm on the emission monochromator. Although excitation at 340 nm gives an increase in the emitted light at 500 nm with increasing $[Ca^{2+}]_i$, excitation at 380 nm gives a decrease in the light output with increasing $[Ca^{2+}]_i$. A ratio of the two outputs therefore gives greater sensitivity, and $[Ca^{2+}]_i$ can be determined directly from these ratios, after subtracting the relative component of the fluorescence that is due to the inherent fluorescence of the cell monolayer, the glass cover-slip, the cuvette and other optics. To determine the extent of autofluorescence, at the end of each experimental run (i.e. for every cover-slip) 2 μM-ionomycin with 2 mM-$MnCl_2$ were added to the cuvette. Mn^{2+} is rapidly translocated across the plasma membrane by ionomycin into the cytoplasm, where it binds much more avidly than Ca^{2+} to fura-2, and completely quenches the fura-2 fluorescence at all wavelengths. The relative quenched values for fluorescence at 340 nm and 380 nm thus represent the autofluorescence values for the system and the cells. We found that ionomycin was a more suitable agent to translocate Mn^{2+} than was digitonin, which (unlike ionomycin) caused a decrease in autofluorescence from monolayers of human umbilical-vein endothelial cells that had not been loaded with fura-2. The corrected fluorescence values with excitation at 340 nm were then divided by those at 380 nm to give a ratio trace. The ratio trace was then processed according to the relation:

$$[Ca^{2+}] = K_d \cdot \frac{(R - R_{min.})}{(R_{max.} - R)} \cdot \frac{S_{f2}}{S_{b2}}$$

K_d for the fura-2/Ca^{2+} complex was taken to be 224 nM at 37 °C [25]. In our system $R_{max.}$ was 19, $R_{min.}$ was 0.9 and S_{f2}/S_{b2} was 10.3. $R_{max.}$ and $R_{min.}$ are the maximal and minimal fluorescence ratios of fura-2 in medium containing saturating $[Ca^{2+}]$ (1 mM), or Ca^{2+}-free, with EGTA. S_{f2} and S_{b2} are the fluorescent values at 380 nm in the absence and in the presence of saturating $[Ca^{2+}]$ respectively.

PGI_2 formation and release was estimated by taking

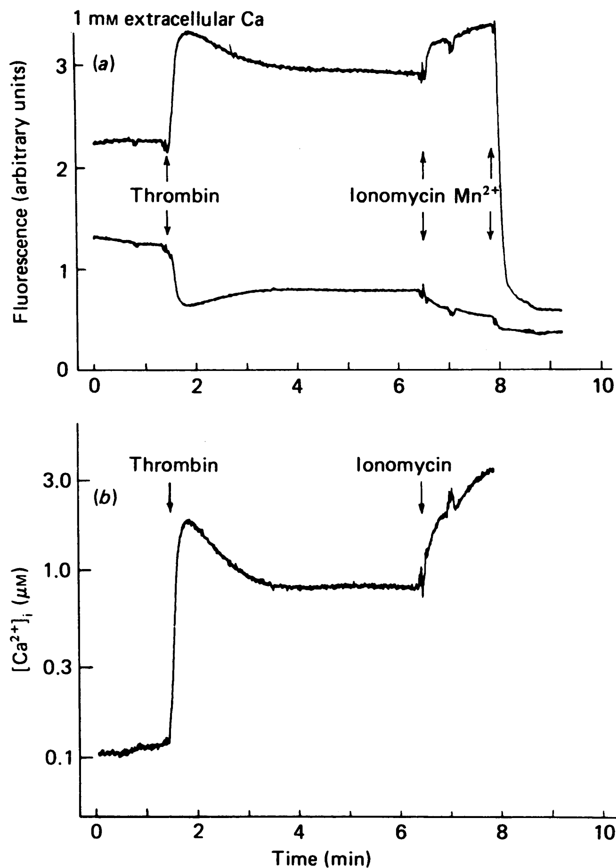

Fig. 1. Stimulated dual-wavelength fluorescence changes of fura-2-loaded human endothelial cells in confluent monolayer

(*a*) Confluent monolayer shows the raw fluorescence intensity emission at 500 nm with 340 nm excitation (top trace) or 380 nm excitation (bottom trace). Thrombin (0.5 unit/ml), ionomycin (2 μM) and $MnCl_2$ (2 mM) were added where indicated. Panel (*b*) shows the calculated $[Ca^{2+}]_i$ trace from the raw data shown in (*a*).

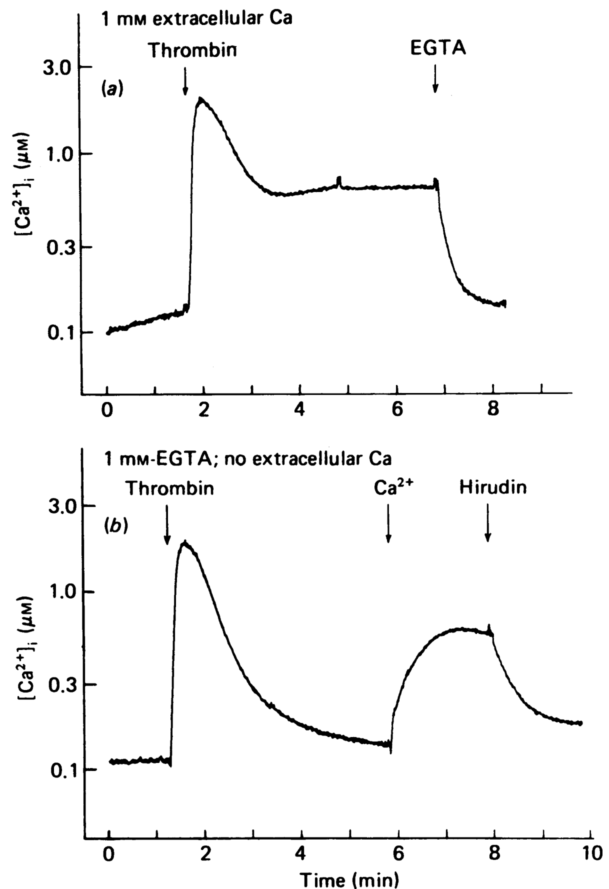

Fig. 2. Effects of manipulating extracellular Ca^{2+} concentration on thrombin-stimulated $[Ca^{2+}]_i$ changes

(*a*) In the presence of 1 mM-Ca^{2+}; thrombin (0.5 unit/ml) and EGTA (2 mM) were added where indicated. (*b*) In the presence of 1 mM-EGTA; thrombin (0.5 unit/ml), $CaCl_2$ (2 mM) and hirudin (5 units/μl) were added where indicated.

small samples of the supernatant at defined time points after stimulation and measuring the amounts of the stable hydrolysis product 6-oxo-$PGF_{1\alpha}$ by radio-immunoassay [26].

For the measurement of inositol phosphate formation, cells were plated on to 12-well tissue-culture plates. When confluent, the cells were fed with Medium 199+20% foetal-calf serum with 10 μCi of [^{3}H]inositol/ml for 2.5 h. The cells were loaded with fura-2 AM by addition from a stock solution and incubated for 45 min. The incubation medium was then removed and replaced with the Hepes-buffered physiological saline described above. Stimulations were initiated by the addition of agonist (50 μl) to 1 ml of medium and stopped by addition of $HClO_4$ (6%, w/v).

Samples were extracted with Freon and trioctylamine, and inositol phosphates were separated by anion-exchange chromatography as described by Downes *et al.* [27].

RESULTS

Thrombin-induced elevations in $[Ca^{2+}]_i$

Fig. 1(*a*) shows the effect of thrombin (0.5 unit/ml) on the fluorescence of human umbilical-vein endothelial cells loaded with fura-2 in the presence of 1 mM extracellular $CaCl_2$. Ionomycin and Mn^{2+} were added at the end of the experiment to estimate the amount of autofluorescence necessary for calibration and calculation of ratios, and hence $[Ca^{2+}]_i$ (see the Methods section). These measurements were obtained with approx. 5×10^4 cells in the light beam. Fig. 1(*b*) shows the calculated $[Ca^{2+}]_i$ values from the data collected in Fig. 1(*a*). Thrombin stimulated a very rapid increase in $[Ca^{2+}]_i$, beginning within 2 s and reaching a peak within 10–15 s. $[Ca^{2+}]_i$ increased about 30-fold, from a resting value of around 100 nM to a peak of 2–3 μM. $[Ca^{2+}]_i$ then declined until a new steady-state value was achieved, of approx. 1 μM, which was maintained for at least 8 min. Removing the source of extracellular Ca^{2+} by adding 2 mM-EGTA during the elevated steady-state phase resulted in a rapid decrease in the $[Ca^{2+}]_i$ to resting pre-stimulated values (Fig. 2*a*).

In the absence of extracellular Ca^{2+} (i.e. with 1 mM-EGTA added before the stimulus), the initial thrombin-stimulated elevation in $[Ca^{2+}]_i$ was very similar to that seen in the presence of extracellular Ca^{2+}, again reaching a peak between 10 and 15 s (Fig. 2*b*). In contrast with the substantial elevated steady-state $[Ca^{2+}]_i$ seen in the presence of extracellular Ca^{2+}, with no extracellular Ca^{2+}

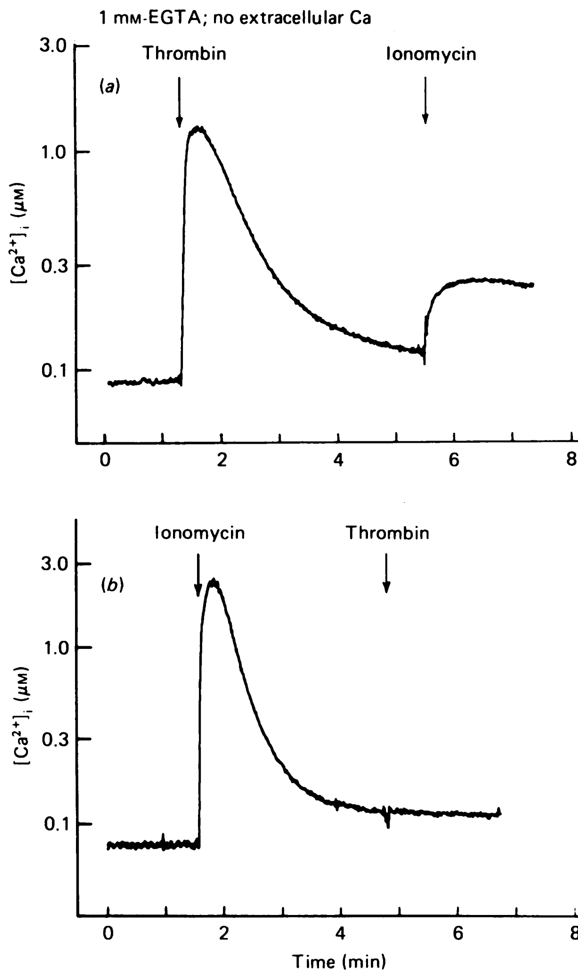

Fig. 3. Effects of thrombin (0.5 unit/ml) and ionomycin (2 μM) on the release of Ca^{2+} from intracellular stores

The experiments were performed in the presence of 1 mM-EGTA.

present $[Ca^{2+}]_i$ rapidly declined to the basal value within 60–90 s. The subsequent addition of 2 mM-$CaCl_2$ (to restore the extracellular free $[Ca^{2+}]$ to about 1 mM) caused a sustained elevation in the $[Ca^{2+}]_i$ to a value very similar to the elevated steady-state value observed after the initial peak obtained in the presence of extracellular free Ca^{2+}. On addition of hirudin, a potent inhibitor of the enzymically active site of thrombin, $[Ca^{2+}]_i$ decreased to the resting value of around 100 nM (Fig. 2*b*). The simplest interpretation of these data is that (*a*) the initial peak is primarily due to the discharge of Ca^{2+} from an intracellular store, (*b*) the maintained elevated $[Ca^{2+}]_i$ observed in the presence of extracellular free Ca^{2+} is caused by the influx of Ca^{2+} across the plasma membrane, and (*c*) this is dependent on the continued presence of the agonist.

Fig. 3(*a*) shows the effect of adding ionomycin (2 μM) to endothelial cells that have already been stimulated with thrombin (0.5 unit/ml) in the absence of extracellular free Ca^{2+}. Under these conditions $[Ca^{2+}]_i$ increased rapidly again to around 0.3 μM, showing that membrane-bounded intracellular stores of Ca^{2+} exist that are not completely dissipated by previous stimulation with thrombin. If the order of additions was reversed (Fig. 3*b*), ionomycin produced a transient elevation in $[Ca^{2+}]_i$ similar to that evoked by thrombin under identical conditions. However, subsequent addition of thrombin produced no further elevation in $[Ca^{2+}]_i$. This is presumably because the intracellular stores cannot reaccumulate Ca^{2+} while ionophore is still present.

Dependence of thrombin-evoked PGI_2 production on extracellular free $[Ca^{2+}]$ and $[Ca^{2+}]_i$

The effects of extracellular free Ca^{2+} on the time-course of 6-oxo-$PGF_{1\alpha}$ accumulation after stimulation with thrombin are shown in Fig. 4. Fig. 4(*a*) shows a typical $[Ca^{2+}]_i$ transient produced on stimulation with thrombin (0.5 unit/ml) in the presence of 1 mM extracellular free Ca^{2+}. The accompanying accumulation of 6-oxo-$PGF_{1\alpha}$ shown gives the mean values from four experiments, including that shown in Fig. 4(*a*). After an initial delay of 10–20 s, there was a marked increase in the rate of PGI_2 synthesis. Although the rate was maximal between 45 and 90 s after the addition of thrombin, 6-oxo-$PGF_{1\alpha}$ continued to accumulate throughout the first 5 min of stimulation. Fig. 4(*b*) shows the results from similar experiments in the absence of extracellular Ca^{2+} with 1 mM-EGTA. Again, after addition of thrombin $[Ca^{2+}]_i$ rose before any increase in 6-oxo-$PGF_{1\alpha}$ was detected. A short delay of 10–20 s was evident, and then the rate of 6-oxo-$PGF_{1\alpha}$ accumulation increased maximally over the following 45–90 s. In contrast with the continued production of 6-oxo-$PGF_{1\alpha}$ in the presence of extracellular Ca^{2+}, in its absence the rate of accumulation of 6-oxo-$PGF_{1\alpha}$ decreased rapidly ~2 min after addition of thrombin, resulting in a final value only 65% of that produced with extracellular Ca^{2+}.

To determine whether thrombin can cause the production and release of PGI_2 by a mechanism other than the stimulated elevation of $[Ca^{2+}]_i$, the cells were depleted of all sources of Ca^{2+}, including agonist-releasable intracellular pools, with ionomycin in the absence of extracellular Ca^{2+}. As shown in Fig. 3, under these conditions thrombin can no longer produce any further elevation in $[Ca^{2+}]_i$. Fig. 5 shows results from experiments in the presence of 1 mM-EGTA, where ionomycin was added followed by thrombin. The time course and extent of the $[Ca^{2+}]_i$ transient and the accumulation of 6-oxo-$PGF_{1\alpha}$ on addition of ionomycin were very similar to those stimulated by thrombin under similar conditions. As expected, addition of thrombin after ionomycin had little, if any, further effect on $[Ca^{2+}]_i$. Likewise, no increase in 6-oxo-$PGF_{1\alpha}$ was detectable, suggesting that PGI_2 production required a rise in $[Ca^{2+}]_i$. To check that lack of thrombin-stimulated PGI_2 synthesis was not because arachidonate-rich phospholipids had been already depleted, extracellular Ca^{2+} was then added. In the continued presence of ionophore and thrombin, $[Ca^{2+}]_i$ rapidly increased to > 1 μM and was accompanied by a further increase in 6-oxo-$PGF_{1\alpha}$. These results strongly suggest that raised $[Ca^{2+}]_i$ is necessary for thrombin-induced PGI_2 production. The lack of effect of thrombin on endothelial cells depleted of their intracellular stores of Ca^{2+} could, however, be due to the experimental conditions, i.e. incubation with ionomycin in medium containing 1 mM-EGTA somehow inhibited the ability of the thrombin receptor to bind or respond to the agonist. To investigate this possibility, measurements of inositol phosphates produced by endothelial cells on stimulation with thrombin were made. Stimulation of fura-2-loaded

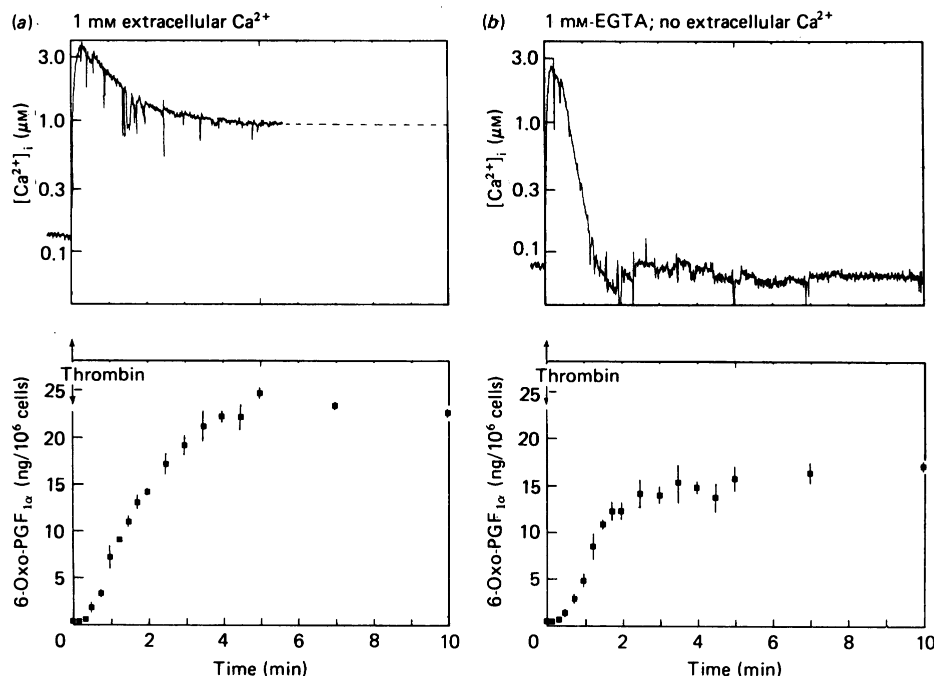

Fig. 4. Effect of extracellular Ca^{2+} on the thrombin-stimulated elevation of $[Ca^{2+}]_i$ and accumulation of 6-oxo-$PGF_{1\alpha}$ in the cuvette

Thrombin (0.5 unit/ml) was added at zero time, (*a*) in the presence of 1 mM-Ca^{2+} or (*b*) in the presence of 1 mM-EGTA. The 6-oxo-$PGF_{1\alpha}$ values are the means ± S.E.M. (error bars) for four experiments; the $[Ca^{2+}]_i$ traces are typical from one of those experiments.

cells with 0.5 unit of thrombin/ml for 15 s in EGTA-containing medium produced a 4.6-fold increase in inositol trisphosphates compared with non-stimulated controls (controls 424 ± 64 d.p.m., $n = 4$; thrombin 1928 ± 95 d.p.m., $n = 4$), presumably because the activated receptor is coupled to and stimulates a phosphatidylinositol 4,5-bisphosphate-specific phospholipase C. When cells were treated with thrombin after 5 min preincubation with ionomycin in the presence of EGTA (by the procedure in Fig. 5), the amounts of inositol trisphosphates increased by 2.3-fold over 15 s compared with cells not treated with thrombin (controls 422 ± 46 d.p.m., $n = 4$; thrombin 959 ± 40 d.p.m., $n = 6$). The results demonstrate that thrombin binds to a functional receptor that successfully couples to stimulate phospholipase C, even after incubation with EGTA and ionomycin where the intracellular stores are discharged and there is no rise in $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ threshold for PGI_2 production

To estimate the threshold $[Ca^{2+}]_i$ required to stimulate the production of PGI_2, in the absence of receptor-mediated responses that may be linked to multiple intracellular activation pathways, endothelial cells were exposed to ionomycin (5 nM–4 μM) in the absence of extracellular Ca^{2+}. The resulting accumulation of 6-oxo-$PGF_{1\alpha}$ after 5 min was measured with the corresponding transient $[Ca^{2+}]_i$ response for each dose of ionomycin. The ■ symbols in Fig. 6 show the relation between 6-oxo-$PGF_{1\alpha}$ concentrations and the peak stimulated $[Ca^{2+}]_i$ values in these experiments. The data show that no PGI_2 formation is stimulated unless $[Ca^{2+}]_i$ reaches a threshold value of between 800 nM and 1 μM. PGI_2 formation increased with increasing $[Ca^{2+}]_i$ above this threshold, up to the limit of resolution of the fluorescent dye, approx. 3 μM.

To test whether the same relationship between $[Ca^{2+}]_i$ and PGI_2 production exists for stimulation with thrombin, similar experiments were performed with concentrations of agonist between 0.002 and 2.0 units/ml. These data are shown in Fig. 6 as □ symbols. No significant difference is apparent between the activation curves obtained in response to ionophore or thrombin. Threshold thrombin doses for effects on $[Ca^{2+}]_i$ and PGI_2 synthesis were approx. 0.05 and 0.25 unit/ml respectively.

DISCUSSION

Recent studies of endothelial cells using fluorescent Ca^{2+} indicators have suggested that $[Ca^{2+}]_i$ can be raised in response to platelet-activating factor, histamine or thrombin by discharge of Ca^{2+} from intracellular stores [15,21,28]. Our results demonstrate that thrombin rapidly discharges Ca^{2+} from an intracellular store that is sufficient to elevate $[Ca^{2+}]_i$ to > 1 μM, and that thrombin also maintains an elevated steady-state $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. The latter is presumably caused by the balance of a stimulated Ca^{2+} influx and plasma-membrane Ca^{2+}-ATPase, which acts to maintain the huge 10000-fold concentration gradient across the plasma membrane.

This conclusion is supported by the finding that removal of extracellular Ca^{2+} during this sustained elevation in $[Ca^{2+}]_i$ caused an immediate fall of $[Ca^{2+}]_i$ to the unstimulated value. This elevated steady-state value could be reconstituted, by adding extracellular Ca^{2+}, after discharging internal stores of Ca^{2+} with thrombin in

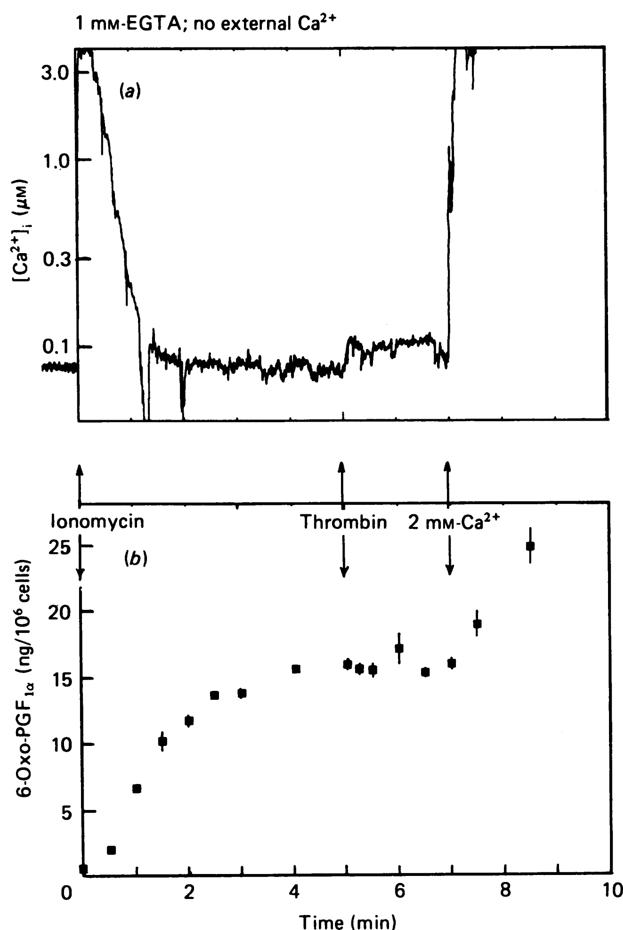

Fig. 5. Effect of thrombin on 6-oxo-PGF$_{1\alpha}$ accumulation from cells depleted of extracellular and intracellular sources of Ca^{2+}

The cells were incubated in the presence of 1 mM-EGTA. Ionomycin (2 μM), thrombin (0.5 unit/ml) and $CaCl_2$ (2 mM) were added where indicated. Panel (*a*) shows a typical $[Ca^{2+}]_i$ trace from one of four experiments, where the simultaneous 6-oxo-PGF$_{1\alpha}$ values are shown as means ± S.E.M. (error bars) in panel (*b*).

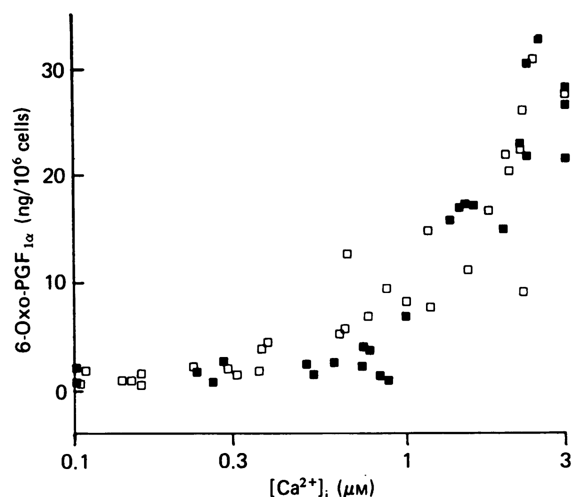

Fig. 6. $[Ca^{2+}]_i$-activation curve for PGI_2 production from intact fura-2-loaded endothelial cells stimulated with ionomycin (■) and thrombin (□)

In the presence of 1 mM-EGTA, samples of cells were stimulated with various doses of ionomycin (5 nM–4 μM) or thrombin (0.002–2 units/ml). The total accumulated amount of 6-oxo-PGF$_{1\alpha}$ was measured after 5 min stimulation and plotted here against the peak stimulated $[Ca^{2+}]_i$.

the absence of extracellular Ca^{2+}. Alternative interpretations of these results, e.g. that removal of extracellular Ca^{2+} with EGTA rapidly depletes an internal store of Ca^{2+} or prevents its fully effective discharge, are unlikely, because the initial transient peak in $[Ca^{2+}]_i$, attributable to release of Ca^{2+} from an internal store, was very similar in the presence or in the absence of extracellular Ca^{2+}. The rapidity with which $[Ca^{2+}]_i$ declined from the elevated steady-state value after removal of active agonist (with hirudin) or removal of extracellular Ca^{2+} (with EGTA) illustrates that the mechanisms for Ca^{2+} extrusion from the cytoplasm, i.e. the plasma-membrane and internal store-membrane Ca^{2+}-ATPases, are well activated under these conditions.

Jaffe *et al.* [17] failed to detect a thrombin-stimulated Ca^{2+} influx into human umbilical-vein endothelial cells. In their study, however, quin2-loaded cells in suspension were used at room temperature. Failure to observe any maintained Ca^{2+} influx may have been due to the lower temperature. It is unlikely that quin2-loaded cells in suspension would account for the difference, because Rotrosen & Gallin [15] detected a substantial Ca^{2+} influx in response to histamine using similar cells. In the study by Jaffe *et al.* [17], analysis of the effect of removing extracellular Ca^{2+} was made complicated by rapidly decaying fluorescence signals, producing a sloping baseline, caused by both settling of cells out of suspension and bleaching of the dye. In our study these problems were avoided by using cells in monolayer, at 37 °C, arguably a more physiologically appropriate state, and by using the far more fluorescent dual-wavelength dye, fura-2, with the added advantage of having far less cytoplasmic Ca^{2+}-buffering.

Thrombin-induced rises in $[Ca^{2+}]_i$, in agreement with the results of Jaffe *et al.* [17], temporally preceded PGI_2 synthesis. In addition we have shown (Fig. 6) that stimulated $[Ca^{2+}]_i$ rises are observed with lower doses of thrombin than stimulated PGI_2 synthesis, again consistent with a causal relationship between the two events. The inability of thrombin to stimulate PGI_2 synthesis when extracellular and intracellular sources of Ca^{2+} were depleted, despite evidence from the inositol phospholipid turnover experiments that the agonist still bound effectively to receptors, demonstrates that raised $[Ca^{2+}]_i$ is necessary for PGI_2 synthesis. The indistinguishable dose–response relationships between $[Ca^{2+}]_i$ and 6-oxo-PGF$_{1\alpha}$ concentrations whether stimulated by ionomycin or thrombin, imply, moreover, that raised $[Ca^{2+}]_i$ is the major, and perhaps the exclusive, regulator of endothelial PGI_2 synthesis in response to thrombin. These data also show that the threshold $[Ca^{2+}]_i$ required to stimulate PGI_2 production is 0.8–1 μM. This value is consistent with the data in Fig. 4, which show that, when $[Ca^{2+}]_i$ is maintained above this threshold for a longer period, PGI_2 synthesis is stimulated for longer. Hence with no external Ca^{2+} the production of PGI_2 was curtailed sooner and the total accumulated 6-oxo-PGF$_{1\alpha}$ after

10 min was correspondingly lower than in the presence of extracellular Ca^{2+}. The concept that a substantial elevation in $[Ca^{2+}]_i$ is needed to induce endothelial PGI_2 synthesis is borne out in experiments under identical conditions where platelet-activating factor was used as the agonist: this caused rises in $[Ca^{2+}]_i$ to 2–3-fold greater than the basal values, but did not lead to PGI_2 release ([28,29]; T. J. Hallam & P. G. Hellewell, unpublished work).

We conclude that elevations in $[Ca^{2+}]_i$ above a critical threshold are necessary for the stimulated production of endothelial PGI_2 in response to thrombin. Our data do not demonstrate where Ca^{2+} acts intracellularly. Release of arachidonate from endothelial membrane phospholipids in response to agonists could involve phospholipase A_2 or the combined actions of phospholipase C and diacylglycerol lipase. Both phospholipases have been shown to be active when endothelial cells are stimulated with bradykinin [30,31]. Phospholipase A_2 is well recognized to be activated by Ca^{2+} [32], although the elegant data of Pollock *et al.* [18,19] show that collagen can stimulate the platelet enzyme without a rise in $[Ca^{2+}]_i$. In endothelial cells and platelets it has been shown that ionophore-induced rises in $[Ca^{2+}]_i$ do not cause activation of phospholipase C [31,33]. In our experiments the activation of phospholipase C by thrombin in cells manipulated to prevent a rise in $[Ca^{2+}]_i$ did not cause PGI_2 production. It is therefore likely that arachidonic acid is liberated by stimulation of phospholipase A_2 by agonist-induced elevation of $[Ca^{2+}]_i$. Activation of phospholipase C generates another second messenger, diacylglycerol, known to mediate cellular responses by activating protein kinase C [34]. Since ionophore and thrombin produced similar $[Ca^{2+}]_i$-activation curves for PGI_2 production, modulation of phospholipase A_2 activity via protein kinase C does not appear to play a significant role in thrombin-stimulated PGI_2 synthesis in endothelium.

We thank the staff of the Delivery Suite, Northwick Park Hospital, for their expert help in the collection of umbilical cords, and Dr. J. L. Gordon for valuable discussion.

REFERENCES

1. Weksler, B. B., Ley, C. W. & Jaffe, E. A. (1978) J. Clin. Invest. **62**, 923–930
2. Baenziger, N. L., Force, L. E. & Becherer, P. R. (1980) Biochem. Biophys. Res. Commun. **92**, 1435–1440
3. Hong, S. L. (1980) Thromb. Res. **18**, 787–795
4. Pearson, J. D., Slakey, L. L. & Gordon, J. L. (1978) Biochem. J. **170**, 265–271
5. Boeynaems, J. M. & Galand, N. (1983) Biochem. Biophys. Res. Commun. **112**, 290–295
6. Levin, R. I., Weksler, B. B., Marcus, A. J. & Jaffe, E. A. (1984) in Biology of Endothelial Cells (Jaffe, E. A., ed.), pp. 228–247, Martinus Nijhoff, Boston
7. Brotherton, A. F. A. & Hoak, J. C. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 495–499
8. Hong, S. L. (1983) Biochim. Biophys. Acta **754**, 258–263
9. Brotherton, A. F. A. (1986) J. Clin. Invest. **78**, 1253–1260
10. Crutchley, D. J., Ryan, J. W., Ryan, V. S. & Fisher, G. M. (1983) Biochim. Biophys. Acta **751**, 99–107
11. Seid, J. M., MacNeil, S. & Tomlinson, S. (1983) Biosci. Rep. **3**, 1007–1015
12. Simpson, A. W. M., Hallam, T. J. & Tink, T. J. (1984) FEBS Lett. **176**, 139–143
13. Hallam, T. J. & Pearson, J. D. (1986) FEBS Lett. **207**, 95–99
14. Hallam, T. J. & Pearson, J. D. (1986) J. Physiol. (London) **337**, 122*P*
15. Rotrosen, D. & Gallin, J. I. (1986) J. Cell Biol. **103**, 2379–2387
16. Luckhoff, A. & Busse, R. (1986) J. Cell. Physiol. **126**, 414–420
17. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) J. Biol. Chem. **262**, 8557–8565
18. Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–877
19. Pollock, W. K., Irvine, R. F. & Rink, T. J. (1986) Eur. J. Pharmacol. **132**, 309–312
20. Hallam, T. J. & Rink, T. J. (1987) in Platelets in Biology and Pathology, vol. 3 (MacIntyre, D. E. & Gordon, J. L., eds.), pp. 353–372, Elsevier, Amsterdam
21. Hamilton, K. K. & Sims, P. J. (1987) J. Clin. Invest. **79**, 600–608
22. Hassid, A. & Oudinet, J.-P. (1986) Prostaglandins **32**, 457–478
23. Jaffe, E. A., Nachman, R. L., Becker, C. G. & Minick, C. R. (1973) J. Clin. Invest. **52**, 2745–2756
24. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) J. Cell Biol. **94**, 325–334
25. Tsien, R. Y., Rink, T. J. & Poenie, M. (1985) Cell Calcium **6**, 145–157
26. Ager, A., Gordon, J. L., Moncada, S., Pearson, J. D., Salmon, J. A. & Trevethick, M. A. (1982) J. Cell. Physiol. **110**, 9–16
27. Downes, C. P., Hawkins, P. T. & Irvine, R. F. (1986) Biochem. J. **238**, 501–506
28. Brock, T. A. & Gimbrone, M. A. (1986) Am. J. Physiol. **250**, H1086–H1092
29. Bussolino, F., Aglietta, M., Sanavio, F., Stacchini, A., Lauri, D. & Camussi, G. (1985) J. Immunol. **135**, 2748–2753
30. Hong, S. L. & Deykin, D. (1982) J. Biol. Chem. **257**, 7151–7154
31. Lambert, T. L., Kent, R. S. & Whorton, A. R. (1986) J. Biol. Chem. **261**, 15288–15293
32. Van den Bosch, H. (1980) Biochim. Biophys. Acta **604**, 191–246
33. Rittenhouse, S. E. & Horne, W. C. (1984) Biochem. Biophys. Res. Commun. **123**, 393–397
34. Nishizuka, Y. (1984) Nature (London) **308**, 693–698

Received 5 October 1987/19 November 1987; accepted 30 November 1987