Differential regulation of von Willebrand factor exocytosis and prostacyclin synthesis in electroporated endothelial cell monolayers

Julie A. FREARSON,*† Paul HARRISON,† Michael C. SCRUTTON* and Jeremy D. PEARSON*§

*Vascular Biology Research Centre, King's College London, Campden Hill Road, London W8 7AH, U.K.

and †Coagulation Research Laboratory, Rayne Institute, UMDS, St. Thomas' Hospital, London, U.K.

We have developed a system to permeabilize human umbilical vein endothelial cells in monolayer culture by application of a high-voltage electric field. The permeabilized preparation allows access of small molecules ($M_r < 1000$) without loss of large cytosolic proteins. Electroporated cells exocytose highly multimeric von Willebrand factor from secretory granules in response to added Ca^{2+} ($\text{EC}_{50} = 0.8 \pm 0.02 \mu\text{M}$), with levels comparable with those observed on stimulation of intact endothelial cells by physiological agonists. MgATP^{2-} potentiates Ca^{2+} -driven von Willebrand factor secretion. Other nucleoside triphosphates, but not non-hydrolysable analogues, can replace

ATP. Electroporated cells also synthesize and release prostacyclin in response to added Ca^{2+} ($\text{EC}_{50} = 0.3 \pm 0.08 \mu\text{M}$), but nucleoside triphosphates markedly inhibit, whereas non-hydrolysable GTP analogues increase, Ca^{2+} -driven prostacyclin synthesis. We conclude that elevation of the intracellular $[\text{Ca}^{2+}]$ is sufficient to cause efficient exocytosis of von Willebrand factor from permeabilized cells, despite evidence that additional second messengers are needed in intact cells. We find no evidence in endothelial cells for a guanine nucleotide-binding protein promoting exocytosis, although one is clearly involved in stimulating Ca^{2+} -driven prostacyclin synthesis.

INTRODUCTION

Von Willebrand factor (vWF) is a large adhesive glycoprotein which plays an important role in the interactions of blood platelets with the blood vessel wall [1]. Cultured vascular endothelial cells secrete vWF both constitutively [2] and via a rapid, regulated pathway from endothelial cell-specific storage granules termed Weibel–Palade bodies [3,4]. Unlike the vWF released by the constitutive pathway, which is almost entirely dimeric, the vWF released by exocytosis from the Weibel–Palade pool is composed entirely of very large multimers [4,5]. Rapid secretion of vWF can be provoked both *in vivo* and *in vitro* by a number of agents, including thrombin, histamine and phorbol 12-myristate 13-acetate (PMA) [6–8]. Stimulation of vWF exocytosis from endothelial cells also results in the plasma membrane expression of P-selectin (GMP-140 or PADGEM), a Weibel–Palade body membrane protein [9] important for the early phase of neutrophil adhesion [10,11].

Endothelial cells also synthesize and release the potent and labile vasodilator prostacyclin (PGI_2). The biosynthesis of PGI_2 is initiated by the intracellular generation of arachidonic acid, which is liberated from phospholipids by an activated phospholipase A_2 (PLA_2). PGI_2 is synthesized and released in response to stimulation by a variety of agents including ATP, bradykinin, histamine, thrombin and the Ca^{2+} ionophore A23187 [12].

In intact endothelial cells thrombin and histamine activate phosphoinositide-specific phospholipase C, resulting in the generation of both inositol 1,4,5-trisphosphate (InsP_3) and *sn*-1,2-diacylglycerol from phosphatidylinositol 4,5-bisphosphate [13,14]. The agonist-induced rise in InsP_3 triggers a rise in cytosolic $[\text{Ca}^{2+}]$ and there are several lines of evidence to suggest that elevated cytosolic Ca^{2+} is necessary both for exocytosis of vWF and synthesis of PGI_2 in endothelial cells [7,15]. However, other agonists, e.g. ATP, which are effective in increasing

cytosolic $[\text{Ca}^{2+}]$ and stimulating the synthesis of PGI_2 are very poor inducers of vWF secretion [16]. Hence other signalling pathways, in addition to an increase in $[\text{Ca}^{2+}]$, appear to be necessary for this response.

We have now directly investigated the relationship between cytosolic $[\text{Ca}^{2+}]$, secretion of vWF and synthesis of PGI_2 , by using an electroporated human umbilical vein cell monolayer preparation. We have defined the conditions under which Ca^{2+} can cause exocytosis of vWF to an extent that is comparable with that observed when intact endothelial cells are stimulated by an effective agonist such as thrombin. We also provide evidence that guanine nucleotide-binding proteins (G-proteins) regulate an effector involved in Ca^{2+} -driven PGI_2 synthesis, but can find no evidence in favour of the involvement of a G-protein mediating Ca^{2+} -stimulated secretion of vWF from these cells.

MATERIALS AND METHODS

Cell culture

Human umbilical vein endothelial cells were isolated and cultured as described by Jaffe et al. [17]. First passage cells were harvested with 0.1% trypsin/0.02% EDTA and plated in 16-mm-diam. wells (Costar) at a concentration of approximately 1×10^5 cells per well, in Medium 199 supplemented with 10% (v/v) fetal-calf serum, 10% (v/v) newborn-calf serum, 90 $\mu\text{g}/\text{ml}$ heparin and 20 $\mu\text{g}/\text{ml}$ endothelial cell growth supplement (prepared from bovine brain [18]). Visually confluent monolayers were formed after overnight incubation and cells were used 96 h after plating.

Electroporation

Confluent cell monolayers were washed twice with 0.5 ml of serum-free Dulbecco's modification of Eagle's medium (DMEM)

Abbreviations used: DMEM, Dulbecco's modification of Eagle's medium; G-protein, guanine nucleotide-binding protein; GDP[S], guanosine 5'-[β -thio]diphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; InsP_3 , inositol 1,4,5-trisphosphate; 6-keto- $\text{PGF}_{1\alpha}$, 6-keto-prostaglandin $\text{F}_{1\alpha}$; PGI_2 , prostacyclin; PLA_2 , phospholipase A_2 ; vWF, von Willebrand factor.

† Present address: Department of Immunology, The Babraham Institute, Babraham, Cambridge CB2 4AT, U.K.

§ To whom correspondence should be addressed.

containing 20 mM Hepes and then transferred into 0.5 ml of permeabilization buffer (280 mM glycine, 20 mM potassium glutamate, 20 mM potassium Hepes, 2 mM magnesium acetate, 2.5 mM potassium EGTA; pH 7.4). Cells in each well were permeabilized using a purpose-built electrode consisting of two stainless steel plates separated by 1 cm. The electrode was partially immersed in permeabilization buffer and suspended approximately 1 mm above the cell monolayer. Each well was subjected to 3×1.5 kV/cm discharges, at 3 μ F capacitance using a Bio-Rad Gene Pulser unit with the electrodes oriented for the second and third discharges at 120° and 240° with respect to the first. This protocol consistently produced monolayers of permeabilized endothelial cells which permitted rapid equilibration of solutes of $M_r < 1000$ into the cytosol, as evidenced by uptake of the fluorescent DNA-binding dye ethidium bromide. There was loss of $> 90\%$ of cellular ATP but $< 10\%$ of the 125000- M_r cytosolic protein lactate dehydrogenase from the population of cells in each well. After 5 min (unless otherwise stated) the permeabilization buffer was removed and 0.6 ml of stimulation buffer added. This was an EGTA/EDTA/ Ca^{2+} buffering system which permitted the manipulation of free $[\text{Ca}^{2+}]$ from approximately 10 nM to 20 μ M (280 mM glycine, 20 mM potassium glutamate, 4 mM magnesium acetate, 20 mM potassium Hepes, 7.5 mM potassium EGTA, 2.5 mM potassium EDTA, $\pm \text{CaCl}_2$ (0 or 8.5 mM), ± 2 mM Mg^{2+} ATP at pH 7.4). Ca^{2+} -free and Ca^{2+} -containing buffers were mixed in different proportions and the free $[\text{Ca}^{2+}]$ calculated using a computer program based on that of Fabiato [19]. After a fixed time (60 min unless otherwise stated) the supernatant fraction was sampled and stored at -20°C before assay for vWF and/or PGI_2 content.

For studies on intact cells, the cells were washed twice in Hepes-containing serum-free DMEM and then maintained in this medium containing vehicle or the appropriate agonist.

Assay for vWF

The vWF content of the supernatant fraction was measured by an enzyme-linked immunosorbent assay [20]. Mouse monoclonal anti-vWF CLBRAg35 [21], used as the coating antibody, was a gift from Dr. J. A. Van Mourik. Samples were assayed in triplicate. The lower limit of detection was 0.5×10^{-3} I.U./ml.

Assay for PGI_2

The PGI_2 content of the supernatant fraction was measured using a specific RIA for 6-keto-prostaglandin $\text{F}_{1\alpha}$ (6-keto- $\text{PGF}_{1\alpha}$), the stable hydrolysis product of PGI_2 [22]. ^{125}I -6-keto- $\text{PGF}_{1\alpha}$ was obtained from Metachem Diagnostics, Nottingham, U.K. Samples were assayed in duplicate. The lower limit of detection was 1 pg/100 μ l.

Multimeric sizing of vWF

For this procedure, cell supernatant fractions were stored in the presence of proteolytic inhibitors (2 μ g/ml leupeptin and pepstatin). Cell lysates were prepared in 0.1 M Tris/HCl (pH 8.0) containing 5 mM EDTA, 1.0% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 2 mM *N*-ethylmaleimide, leupeptin (2 μ g/ml) and pepstatin (2 μ g/ml). Samples (5 μ l) were electrophoresed on 0.8% (w/v) high-gelling-temperature SDS-agarose. They were capillary blotted on to 0.45- μ m-pore-size nitrocellulose and multimers were visualized by indirect immunostaining with alkaline phosphatase-coupled secondary antibody [23].

ATP measurements

Cells were washed twice in phosphate-buffered saline before adding 0.3 ml of 5% trichloroacetic acid. Samples were neutralized with 0.45 ml of 0.5 M tri-*n*-octylamine in freon and the upper aqueous phase was collected and stored at -20°C before measurement of ATP content. Reversed-phase HPLC was performed on a 25 cm $\times$ 0.46 cm column (5 μ m ODS-Hypersil). Samples (0.1 ml) were injected and eluted at 1 ml/min with 0.05 M $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 6.6) at room temperature according to the method of Gordon et al. [24].

Assay for lactate dehydrogenase activity

Lactate dehydrogenase activity in cell-conditioned permeabilization or stimulation buffer and in cell lysates prepared in 0.1% Triton X-100 was measured using a modification of the method of Keiding et al. [25]. Samples (50 μ l) were diluted into 3.1 ml of assay buffer (56 mM Tris, 5.6 mM EDTA, pH 7.4, containing 0.2 μ M NADH and 45 mM sodium pyruvate) at 37°C and mixed in a quartz cuvette. The initial rate of change in absorbance at 340 nm was recorded, reflecting NAD production, over 5 min. The lactate dehydrogenase activity in the supernatant fraction was expressed as a percentage of the total activity in the well. Activity was always assayed within 10 min of sample generation.

RESULTS

Figure 1 shows an example of the dose-related secretion of vWF and release of PGI_2 from electroporated cells in response to $[\text{Ca}^{2+}]$. When $[\text{Ca}^{2+}]$ was increased from 0.05 μ M to 5 μ M, vWF and PGI_2 release both increased in a dose-dependent manner, to levels respectively 5.0 ± 0.5 - and 2.9 ± 0.2 -fold (mean $\pm$ S.E.M., six experiments) greater than when cells were incubated without added Ca^{2+} . The Ca^{2+} content of the nominally

Figure 1 Dependence of vWF secretion and PGI_2 synthesis on $[\text{Ca}^{2+}]$ in electroporated endothelial cells

Electroporated cells were exposed to varying $[\text{Ca}^{2+}]$. After 60 min the vWF (●) and 6-keto- $\text{PGF}_{1\alpha}$ (■) released into the supernatant fraction were assayed. Values obtained in the presence of Ca^{2+} were expressed as percentages of those obtained with EGTA/EDTA alone (nominally Ca^{2+} -free). Control release (100%) was 0.5 ± 0.03 munit of vWF and 0.13 ± 0.02 ng of 6-keto- $\text{PGF}_{1\alpha}$ per well. Lactate dehydrogenase (LDH) release into the supernatant fraction (▼) was also determined at the indicated $[\text{Ca}^{2+}]$. Data points are means $\pm$ S.E.M. of four observations from a typical experiment which is representative of five others showing similar results.

Figure 2 Time course of Ca^{2+} -driven vWF secretion and PGI_2 synthesis

Electropermeabilized cells were exposed to $1 \mu\text{M}$ Ca^{2+} for up to 60 min. Levels of vWF (●) and PGI_2 (■) in the supernatant fraction were assayed. Values obtained in the presence of Ca^{2+} were expressed as percentages of time-matched controls to which no Ca^{2+} was added. Data points are means $\pm$ S.E.M. from three experiments.

Ca^{2+} -free buffer was estimated as approximately 10 nM. The EC_{50} value for Ca^{2+} in vWF secretion was $0.8 \pm 0.2 \mu\text{M}$ and that for Ca^{2+} in PGI_2 synthesis was $0.3 \pm 0.08 \mu\text{M}$ (mean $\pm$ S.E.M., six experiments). Lactate dehydrogenase activity in the supernatant fraction 30 min after stimulation at 10 nM Ca^{2+} was $5.1 \pm 0.2\%$ of total cellular activity and at $10 \mu\text{M}$ Ca^{2+} was $4.7 \pm 0.2\%$ (mean $\pm$ S.E.M., three experiments), indicating that there was no concurrent release of cytosolic proteins.

Figure 2 illustrates the relative time courses of secretion of vWF and synthesis and release of PGI_2 from permeabilized cells in the presence of $1 \mu\text{M}$ Ca^{2+} . Significant stimulation of vWF secretion by $1 \mu\text{M}$ Ca^{2+} was apparent within 5 min of exposure to Ca^{2+} ($P < 0.001$) and continued linearly for 30 min. In contrast a marked increase in PGI_2 synthesis was detectable within 2 min, reaching a peak at 5 min after which no further Ca^{2+} -driven synthesis was observed. Ca^{2+} levels had to be elevated for > 30 min (for vWF) and 5 min (for PGI_2) to achieve the full extent of Ca^{2+} -driven synthesis (data not shown).

Within 5 min of permeabilization these monolayers contained $< 10\%$ of their initial ATP levels (21.2 ± 0.4 mM assuming a cell volume of 1 pl). In several cell types it has been deduced that MgATP^{2-} is essential for Ca^{2+} -dependent exocytosis. The data in Figures 1 and 2 show Ca^{2+} -stimulated secretion of vWF in the absence of added MgATP^{2-} . This secretion was maintained even when cellular [ATP] was reduced to $< 2\%$ (below levels of detection) of that of intact cells using metabolic inhibitors (data not shown). However, Table 1 shows that Ca^{2+} -driven secretion of vWF was significantly enhanced in the presence of 2 mM MgATP^{2-} . The extent of secretion stimulated by $1 \mu\text{M}$ Ca^{2+} from permeabilized cells was directly compared with that for agonist-driven secretion from intact cells (Table 1). When permeabilized cells were stimulated with $1 \mu\text{M}$ Ca^{2+} alone the extent of vWF secretion was approximately half that secreted from intact cells

Table 1 Comparison of vWF secretion from permeabilized and intact endothelial cells

Electropermeabilized cells were exposed to ~ 10 nM Ca^{2+} (no added Ca^{2+}), or $1 \mu\text{M}$ Ca^{2+} in the presence or absence of 2 mM MgATP^{2-} . Intact cells were incubated with vehicle or maximal doses of PMA, histamine or thrombin. After 60 min the vWF in the supernatant fraction was assayed. Data are expressed as percentages of total cell vWF pool. Bars show means $\pm$ S.E.M. from 4 to 8 experiments. * $P < 0.05$ versus $1 \mu\text{M}$ Ca^{2+} alone, by unpaired Student's t test.

Stimulus	% of total cell vWF	
	Permeabilized	Intact
10 nM Ca^{2+}	3.61 ± 0.8	—
10 nM Ca^{2+} + ATP	5.2 ± 1.2	—
$1 \mu\text{M}$ Ca^{2+}	15.1 ± 3.0	—
$1 \mu\text{M}$ Ca^{2+} + ATP	$32.6 \pm 7.0^*$	—
None	—	3.56 ± 0.9
40 nM PMA	—	24.6 ± 4.0
10 nM Thrombin	—	22.0 ± 6.0
$10 \mu\text{M}$ Histamine	—	18.0 ± 7.0

Figure 3 Multimeric analysis of vWF released from intact and electropermeabilized cells

Electropermeabilized cells were exposed to $1 \mu\text{M}$ Ca^{2+} in the presence (lane F) or the absence (lanes C, D and E) of 2 mM MgATP^{2-} . Intact cells were incubated with 25 nM thrombin (lane G). After 60 min the supernatants were collected and 5 μl of each were run on 0.8% agarose gels, blotted on to nitrocellulose and immunostained [23]. Lane A, normal pooled human serum; lanes B and H, cell lysates.

in response to maximally effective doses of PMA, thrombin or histamine. When permeabilized cells were stimulated with $1 \mu\text{M}$ Ca^{2+} + 2 mM MgATP^{2-} , vWF secretion was not significantly different from that observed in agonist-stimulated intact cells.

The nature of the vWF species being released from permeabilized cells, and hence their intracellular source, was defined by analysis of the multimeric composition of the secreted vWF. Lane A in Figure 3 shows the characteristic series of increasing-molecular-mass multimers found in serum. Lanes B and H illustrate total endothelial cell vWF, consisting only of very-high- M_r multimers and low- M_r dimers, respectively characteristic of the regulated and constitutive pathways of release. Lane G shows the vWF released from intact cells on stimulation with thrombin, which is entirely high- M_r multimers. The material

Figure 4 Potentiation of Ca^{2+} -driven vWF secretion by ATP

Electroporated cells were exposed to $0.5 \mu\text{M}$ Ca^{2+} with various concentrations of MgATP^{2-} . After 60 min the vWF in the supernatant fraction was assayed. Release in the absence of ATP was $310 \pm 26\%$ of control. Control release (no added Ca^{2+}) was 1.5 ± 0.1 munit/well. Data points are means $\pm$ S.E.M. from four experiments. vWF secretion was significantly ($P < 0.05$) enhanced at 0.75 mM MgATP^{2-} .

released from permeabilized cells, in the presence (lane F) or in the absence (lanes C, D and E) of MgATP^{2-} contained only high- M_r vWF multimers, demonstrating that the vWF secreted from permeabilized cells on stimulation by Ca^{2+} originated from the Weibel-Palade bodies.

The MgATP^{2-} concentration required to observe potentiation of Ca^{2+} -driven vWF release was in the millimolar range (Figure 4) with an EC_{50} value of $0.70 \pm 0.01 \text{ mM}$ (mean $\pm$ S.E.M., four experiments). Figure 5(a) shows that addition of MgATP^{2-} significantly enhanced the extent of secretion observed in the presence of 0.05 to $5 \mu\text{M}$ Ca^{2+} and reduced the EC_{50} for Ca^{2+} from $0.8 \pm 0.2 \mu\text{M}$ to $0.3 \pm 0.1 \mu\text{M}$ (mean $\pm$ S.E.M., three experiments). In contrast Ca^{2+} -driven PGI_2 synthesis was significantly inhibited by the addition of 2 mM MgATP^{2-} (Figure 5b).

The mechanism by which MgATP^{2-} modulates these Ca^{2+} -driven responses was investigated with the use of alternative nucleotides and their non-hydrolysable analogues (Table 2). The ability of nucleotides to potentiate Ca^{2+} -driven vWF secretion was dependent on their ability to donate a phosphate. Thus, the triphosphate nucleotides each potentiated secretion ($\text{ATP} = \text{GTP} > \text{CTP}$) but diphosphates (ADP) and non-hydrolysable analogues [β, γ -methylene-ATP, guanosine 5'-[γ -thio]triphosphate (GTP[S]) and β, γ -methylene-GTP] were ineffective. In contrast to reports in other permeabilized cells (see the Discussion), Ca^{2+} -dependent secretion in endothelial cells was not modulated by activators (GTP[S], β, γ -methylene-GTP) or inhibitors [guanosine 5'-[β -thio]diphosphate (GDP[S])] of heterotrimeric GTP-binding protein function. Furthermore, GDP[S] did not attenuate the effects of ATP or GTP on Ca^{2+} -driven secretion (data not shown).

In contrast GTP[S] and β, γ -methylene-GTP were equally effective potentiators of Ca^{2+} -driven PGI_2 synthesis in these cells (Table 2), whereas all the hydrolysable nucleoside triphosphates

Figure 5 Effect of MgATP^{2-} on Ca^{2+} -driven vWF secretion and PGI_2 synthesis

Electroporated cells were exposed to various $[\text{Ca}^{2+}]$ alone (open symbols) or in the presence of 2 mM MgATP^{2-} (closed symbols). After 60 min vWF (a) and 6-keto- $\text{PGF}_{1\alpha}$ (b) in the supernatant fraction were assayed. Control release was 0.88 ± 0.25 munit of vWF and $0.27 \pm 0.01 \text{ ng}$ of 6-keto- $\text{PGF}_{1\alpha}$ per well. Data points are means $\pm$ S.E.M. from three experiments. Ca^{2+} -driven release of vWF was significantly ($P < 0.001$) higher, and that of PGI_2 significantly lower ($P < 0.01$), in the presence of MgATP^{2-} .

tested significantly inhibited PGI_2 synthesis, though GTP and CTP were considerably less effective than ATP. None of the nucleotides tested induced significant vWF secretion or PGI_2 synthesis in the absence of added Ca^{2+} (data not shown).

Figure 6 illustrates further the markedly different effects of GTP[S] on Ca^{2+} -driven PGI_2 synthesis and vWF secretion. GTP[S] dose-dependently potentiated PGI_2 synthesis stimulated by micromolar Ca^{2+} . Addition of $300 \mu\text{M}$ GTP[S] caused a maximal 2.8 ± 0.1 -fold (mean $\pm$ S.E.M., $n = 6$) increase over the response to Ca^{2+} alone. This potentiation was reversed by co-incubation with excess GDP[S] (Figure 6). Across the range of doses tested GTP[S] had no significant effect on the ability of Ca^{2+} to stimulate vWF secretion.

Table 2 Effect of various nucleotides on Ca^{2+} -driven vWF secretion and PGI_2 synthesis in electroporated human umbilical vein endothelial cells

Electroporated cells were exposed to $0.3 \mu\text{M}$ Ca^{2+} alone, or in the presence of the indicated nucleotides (2 mM). After 60 min vWF and 6-keto- $\text{PGF}_{1\alpha}$ in the supernatant fraction were assayed. Control release was 0.92 ± 0.11 munit of vWF and 0.13 ± 0.01 ng of 6-keto- $\text{PGF}_{1\alpha}$ per well. Data are means $\pm$ S.E.M. from three experiments. $**P < 0.001$, $*P < 0.01$ relative to Ca^{2+} alone. Abbreviation: nd, not determined.

Added nucleotide (2 mM)	Response compared with 10 nM Ca^{2+} (%)	
	vWF	6-keto- $\text{PGF}_{1\alpha}$
None	165 ± 13	150 ± 9
ATP	$323 \pm 25^{**}$	$108 \pm 6^*$
ADP	190 ± 20	nd
β, γ -Methylene-ATP	186 ± 11	161 ± 21
GTP	$302 \pm 18^{**}$	$134 \pm 2^*$
GTP[S]	163 ± 10	$270 \pm 14^{**}$
β, γ -Methylene-GTP	163 ± 17	$315 \pm 12^{**}$
GDP[S]	178 ± 10	189 ± 9
CTP	$218 \pm 13^*$	$135 \pm 2^*$

Figure 6 Effect of GTP[S] on Ca^{2+} -stimulated PGI_2 synthesis and vWF secretion from electroporated endothelial cells

Electroporated cells were exposed to $0.3 \mu\text{M}$ Ca^{2+} in the presence of various concentrations of GTP[S] (open symbols), and also to $100 \mu\text{M}$ GTP[S] + 1 mM GDP[S] (closed symbol). After 60 min 6-keto- $\text{PGF}_{1\alpha}$ ($\square$, $\blacksquare$) and vWF ($\circ$) levels in the supernatant fraction were assayed. Control release (zero added Ca^{2+}) was 0.15 ± 0.03 ng of 6-keto- $\text{PGF}_{1\alpha}$ and 0.67 ± 0.05 munit of vWF per well. In the presence of Ca^{2+} alone 6-keto- $\text{PGF}_{1\alpha}$ release was $221 \pm 15\%$ and vWF release was $250 \pm 10\%$ of control. Data points are means $\pm$ S.E.M. from six experiments. $**P < 0.01$ for GDP[S] + GTP[S] versus GTP[S] alone.

DISCUSSION

Regulated secretion from eukaryotic cells requires the translocation of membrane-bound granules within the cytoplasm and fusion with the plasma membrane. The synthesis of PGI_2 results from the liberation of arachidonic acid from membrane phospholipids via the activation of PLA_2 [12]. Both these events are

controlled by agonist-induced generation of second messengers. Evidence obtained to date supports a central role for Ca^{2+} in the regulation of PGI_2 synthesis in endothelial cells in response to agonists such as thrombin and ATP [15,26]. In contrast, studies in intact endothelial cells have not established a quantitative link between raised cytosolic $[\text{Ca}^{2+}]$ and vWF secretion, although the involvement of Ca^{2+} in mediating agonist-induced vWF secretion has been demonstrated by: (1) the ability of intracellular Ca^{2+} chelators to inhibit secretion in response to histamine or thrombin [8,27]; (2) the requirement of millimolar extracellular Ca^{2+} for agonist-induced vWF secretion [7]; and (3) stimulation of vWF secretion by the Ca^{2+} ionophore A23187 [7]. In addition, Birch et al. [27] concluded from studies using inhibitors in detergent-permeabilized cells that Ca^{2+} and calmodulin were necessary for thrombin-induced vWF secretion. However, several other Ca^{2+} -mobilizing agonists, e.g. ATP and bradykinin, are weak or ineffective agonists for vWF secretion, and A23187 and phorbol esters that activate protein kinase C act synergistically [16]. Hence signalling pathways, as yet undefined (since selective inhibitors of protein kinase C do not inhibit vWF secretion induced by histamine or thrombin [16]), are required in addition to those utilizing raised cytosolic $[\text{Ca}^{2+}]$ to produce a full response to physiological agonists.

Chemical or mechanical permeabilization techniques to manipulate cytosolic components have been used previously to investigate stimulus-secretion coupling in several cell types, including endothelial cells [27–30]. We have used electroporation of endothelial cell monolayers to measure vWF secretion and PGI_2 synthesis, under conditions in which cytosolic $[\text{Ca}^{2+}]$ is directly controlled by external $[\text{Ca}^{2+}]$ but there is minimal loss of cell proteins.

The Ca^{2+} dose-response curve for vWF secretion in electroporated endothelial cells (Figure 1) is similar to those reported for exocytosis from other permeabilized cell systems [31]. It does, however, differ significantly from that recently described for Ca^{2+} -driven vWF secretion from detergent-permeabilized endothelial cells, where Ca^{2+} -driven secretion began at $1 \mu\text{M}$ and continued to rise beyond $10 \mu\text{M}$, with an approximate EC_{50} value of $3 \mu\text{M}$ [27]. Ca^{2+} -driven secretion and synthesis of PGI_2 in the electroporated system (Figure 1) also occurs over the range of $[\text{Ca}^{2+}]$ normally attained on stimulation of intact cells by agonists such as thrombin or ATP [15,26]. The supraphysiological requirement for Ca^{2+} in detergent-permeabilized cells may be a consequence of damage or inactivation of intracellular transduction or cytoskeletal elements.

The vWF secreted from electroporated cells in response to Ca^{2+} originated from the Weibel-Palade bodies, since it consisted entirely of large multimers (Figure 3). Furthermore, because addition of micromolar Ca^{2+} caused no increase in release of the cytosolic enzyme lactate dehydrogenase, which is smaller than the released vWF multimers, the response observed was an exocytotic event and was not due to granule destabilization in the cytosol.

The time course for stimulated vWF secretion (Figure 2) is in good agreement with that reported for agonist-stimulated secretion from intact endothelial cells [6,8]. It should be noted, though, that in the permeabilized cells $[\text{Ca}^{2+}]$ had to be maintained at an elevated level throughout the period of secretion, since removal of Ca^{2+} before secretion was complete resulted in a less than maximal response. The rapid and transient nature of PGI_2 synthesis in electroporated cells correlates well with that observed in intact endothelial cells stimulated with agonists, even though $[\text{Ca}^{2+}]$ was elevated for longer than occurs in intact cells.

ATP and GTP were equally effective in potentiating Ca^{2+} -

driven vWF secretion (Table 2), as found in other permeabilized secretory cell systems [32,33]. In some cell types GTP (or a non-hydrolysable analogue) appears to be an essential co-factor for Ca^{2+} -driven secretion [33,34]. It has therefore been proposed that, in these cell types, ATP acts to promote secretion by maintaining cellular levels of GTP by nucleotide transphosphorylation, thus allowing activation of a G-protein (G_{β}) believed to be directly involved in exocytosis. In the majority of permeabilized cell types, however, GTP and its analogues serve to modulate rather than to initiate Ca^{2+} -stimulated secretion [32,35,36]. Our results argue against the involvement of G_{β} in the secretion of vWF from endothelial cells, since neither activators nor inhibitors of G-protein function had any significant effect on Ca^{2+} -driven secretion (Table 2). The effects of ATP and GTP on vWF secretion depend on their ability to donate phosphate, since neither non-hydrolysable analogues nor ADP can support increased Ca^{2+} -driven secretion (Table 2). It is therefore likely that ATP, GTP (and CTP) potentiate Ca^{2+} -driven secretion of vWF by stimulating kinase activity, either directly or via nucleotide transphosphorylation to maintain levels of ATP.

In many secretory systems addition of ATP is necessary to observe Ca^{2+} -driven exocytosis. In several cell types the requirement for added ATP appears to be absolute [37–41], but in others a significant extent of the Ca^{2+} -driven response can be observed in the absence of added ATP [42–44]. In endothelial cells approximately half-maximal Ca^{2+} -driven vWF secretion can be observed despite a depletion in cellular [ATP] to < 0.4 mM (Table 1, Figure 4). This can occur if the intact cell contains a subset of granules that have already been primed for secretion by a process which is dependent on ATP [43–45]. The ATP-dependent reaction in this process has yet to be defined, but it has a K_m value greater than those reported for protein kinases [46–48].

PGI_2 synthesis occurs at lower $[\text{Ca}^{2+}]$ in permeabilized than in intact endothelial cells (compare data here with those in [15]). This may be due to the depletion of nucleotide levels in the permeabilized cells, since addition of ATP decreased the potency of Ca^{2+} to stimulate PGI_2 synthesis (Figure 5). By comparison, GTP and CTP were considerably less effective. As for stimulation of vWF release, ATP is likely to affect PGI_2 synthesis via the donation of a phosphate group since β, γ -methylene-ATP was ineffective. However, in contrast to their effects on vWF secretion, GTP[S] or β, γ -methylene-GTP greatly stimulated Ca^{2+} -driven PGI_2 synthesis (Table 2, Figure 6). This is apparently due to the activation of a G-protein(s) since it was specific for guanine nucleotide analogues and was reversed by GDP[S]. PGI_2 synthesis in intact endothelial cells in response to some agonists is pertussis toxin-sensitive, indicating the involvement of a heterotrimeric G-protein [49,50], although the point at which it acts in the pathway has not been fully elucidated. Phospholipase C in endothelial cells is G-protein-linked [51,52]; there is also evidence in a variety of (non-endothelial) cell types for the G-protein regulation of PLA_2 [53,54]. Our results imply G-protein coupling with the Ca^{2+} -sensitive effector in PGI_2 synthesis, almost certainly cytosolic PLA_2 [55], which may become associated with a G-protein on its Ca^{2+} -mediated translocation from cytosol to membrane phospholipids.

In summary, Ca^{2+} can stimulate exocytosis of vWF from permeabilized endothelial cells as effectively as do receptor-linked agonists such as thrombin and histamine in intact cells, despite the evidence that additional second messengers are involved in the responses to agonists. Whether this implies that Ca^{2+} acts at more than one point in the secretory pathway requires further study. Elevation of $[\text{Ca}^{2+}]$ throughout the incubation period was required to achieve full secretion of vWF

from permeabilized cells, which may reflect an early need for raised intracellular $[\text{Ca}^{2+}]$ to activate further second messengers (e.g. calmodulin or protein kinase C) plus a continued need for extracellular Ca^{2+} acting at the site of the secretory fusion event. Approximately half of the vWF pool sensitive to secretion by agonists can be released by Ca^{2+} when ATP levels are reduced by as much as 98 %, but the remainder depends on phosphorylation of a protein (or proteins) required for exocytosis. These may prove to be either regulators (e.g. second messenger proteins) or essential components of the fusion machinery. In contrast to the majority of secretory cell studies there is no evidence for the existence of a G-protein associated with exocytosis in endothelial cells. There is, however, clear evidence that a G-protein-coupled effector modulates PGI_2 synthesis in these cells.

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