The roles of protein kinase C and intracellular Ca^{2+} in the secretion of von Willebrand factor from human vascular endothelial cells

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Secretion of von Willebrand factor (vWf) glycoprotein from storage granules in human umbilical-vein endothelial cells was studied *in vitro*. Either elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) with a Ca^{2+} ionophore or activation of protein kinase (PK) C by phorbol 12-myristate 13-acetate caused vWf secretion, and together the agents acted synergistically. However, when vWf release was stimulated by receptor-mediated agonists, selective inhibition of PKC had no effect on histamine-induced secretion and significantly elevated thrombin-induced secretion. Furthermore, ATP, which efficiently elevates $[\text{Ca}^{2+}]_i$ in these cells, was a very poor effector of vWf release. We conclude that elevation of $[\text{Ca}^{2+}]_i$ by physiological agonists is necessary for vWf release, but other signalling mechanisms, as yet uncharacterized, but not due to PKC activation, are required for full induction of the secretory pathway.

INTRODUCTION

Endothelial cells are the major source of plasma von Willebrand factor (vWf). vWf is also detected in the culture medium of human vascular endothelial cells *in vitro* (Jaffe *et al.*, 1974), and hence these cells have been used extensively to study the biosynthesis, storage and secretion of vWf. By using this system, secretion of vWf has been shown to occur by two different pathways. Most of the newly synthesized vWf is secreted constitutively, and consists mainly of incompletely processed low-molecular-mass multimers (Loesberg *et al.*, 1983; Sporn *et al.*, 1986), whose probable function *in vivo* is the maintenance of a constant level of plasma vWf to serve as the carrier protein for coagulation Factor VIII. Such basal release is directly linked to protein synthesis *de novo*.

Additional acute release of preformed vWf is induced by a range of stimuli. The vWf protein released by this second pathway consists of high-molecular-mass multimers, which have been shown to bind more avidly than the lower-molecular-mass multimers to both blood platelets and the endothelial-cell extracellular matrix, where they provide an important cofactor for platelet adhesion to the damaged vessel wall (Ruggeri & Zimmerman, 1980; Sporn *et al.*, 1986, 1987). Agonist-induced vWf release is associated with the secretion of vWf from intracellular granules (Weibel–Palade bodies), identified as the endothelial-cell storage site for vWf (Wagner *et al.*, 1982; Reinders *et al.*, 1984; Ewenstein *et al.*, 1987). Agonists of vWf release from cultured endothelium include thrombin (Levine *et al.*, 1982), histamine (Hamilton & Sims, 1987), fibrin (Ribes *et al.*, 1987), Ca^{2+} ionophore A23187 and 4β -phorbol 12-myristate 13-acetate (PMA) (Loesberg *et al.*, 1983; Reinders *et al.*, 1984, 1987) and tumour-secreted vascular permeability factor (Brock *et al.*, 1991).

All of the agonists of vWf secretion increase intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), except PMA, which lowers agonist-stimulated $[\text{Ca}^{2+}]_i$,

(Carter *et al.*, 1989). Stimulated, but not basal, vWf secretion is dependent on the presence of extracellular Ca^{2+} (Loesberg *et al.*, 1983). It is, however, not clear to what extent receptor-mediated secretion of vWf is due to elevations of $[\text{Ca}^{2+}]_i$ or activation of protein kinase (PK) C.

In the present study we have used four inhibitors of PKC and an activator, PMA, to investigate the extent to which the kinase mediates vWf release in response to the agonists thrombin and histamine. Two relatively poorly selective PK inhibitors, staurosporine and H-7, were compared with two novel inhibitors, Ro 31-7549 and Ro 31-8220, with much greater selectivity for PKC (Davis *et al.*, 1989). Additionally, combinations of agonists were used to explore further the signal-transduction mechanisms involved in the vWf secretory response.

METHODS AND MATERIALS

Cell culture

Human umbilical-vein endothelial cells were isolated and cultured as described by Jaffe *et al.* (1973). First-passage cells were harvested with 0.1% trypsin/0.02% EDTA and plated in 16 mm-diameter wells (Gibco, Uxbridge, Middx., U.K.), at a concentration of approx. 8×10^4 cells per well, in medium 199 supplemented with 10% (v/v) fetal-calf serum, 10% (v/v) newborn-calf serum, 90 μg of heparin/ml and 20 μg endothelial-cell growth supplement prepared from bovine brain (Maciag & Weinstein, 1984)/ml. Visually confluent monolayers were formed after overnight incubation, and cells were used 72–96 h after harvest.

For the experiments, cells were transferred into 1.0 ml of serum-free medium 199, containing 20 mM-Hepes. After 60 min, 0.5 ml of the medium was removed and replaced with an equal volume of fresh medium, containing the appropriate agonist or combination of agonists, for a further 60 min. In certain experiments, 1.0 ml of the medium was removed and cells were

Abbreviations used: $[\text{Ca}^{2+}]_i$, concn. of cytosolic Ca^{2+} ; DMSO, dimethyl sulphoxide; PMA, 4β -phorbol 12-myristate 13-acetate; PK, protein kinase; vWf, von Willebrand factor.

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pretreated for 5 min with a PK inhibitor, before challenge with an agonist. The inhibitor was present at the stated concentration throughout the stimulatory period. Cell supernatants were then removed and stored at -20°C before assay for vWf content.

Assay for vWf

Release of vWf into the cell supernatant was measured by an enzyme-linked immunosorbent assay as described previously (Paleolog *et al.*, 1990). Mouse monoclonal anti-vWf CLBRAg35 (Stel *et al.*, 1984), used as the coating antibody, was a gift from Dr. J. A. Van Mourik. Samples were assayed in triplicate, and the lower limit of detection was 2.5×10^{-4} I.U./ml.

Materials

Thrombin (from human plasma; activity 3000 NIH units/mg of protein), histamine dihydrochloride, PMA, ATP (disodium salt) from horse muscle, bradykinin acetate and Ca^{2+} ionophore A23187 (free acid) were all purchased from Sigma Chemical Co., Poole, Dorset, U.K. Staurosporine was from Calbiochem, Nottingham, U.K.; H-7 (hydrochloride salt) was from Semat Technical (UK) Ltd., St. Albans, Herts., U.K.; and Ro 31-7549 and Ro 31-8220 (compounds 2 and 3 respectively in Davis *et al.*, 1989) were gifts from Dr. T. Hallam, Roche Products, Welwyn Garden City, Herts., U.K.

Stock solutions of PMA, A23187, staurosporine, H-7, Ro 31-7549 and Ro 31-8220 were in dimethyl sulphoxide (DMSO). The concentration of DMSO in the experiments did not exceed 0.1 % (v/v), and alone this vehicle had no effect on vWf secretion. Thrombin was initially dissolved in distilled water. Solutions of ATP and histamine were made up directly in medium 199.

RESULTS

Effects of PK inhibitors on vWf secretion

Maximally effective doses of each of the three agonists (thrombin, 2.5 units/ml; histamine, $10 \mu\text{M}$; PMA, 40 nM; Levine *et al.*, 1982; Hamilton & Sims, 1987; Paleolog *et al.*, 1991) were used in these experiments. None of the PK inhibitors significantly affected constitutive vWf release.

The vWf secretory response induced by PMA was decreased, in a dose-dependent manner, by each PK inhibitor. Staurosporine (50–100 nM) decreased PMA-induced vWf release by $> 80\%$, and in two of four experiments there was no significant release above basal (Fig. 1). Ro 31-8220 mimicked the inhibitory profile of staurosporine, whereas Ro 31-7549 was slightly less potent (Fig. 1). An inhibitory effect of H-7 was seen only at $50 \mu\text{M}$ (the highest concentration used), when it decreased the response to PMA by 54 % (results not shown).

Thrombin-stimulated vWf release was unaffected by up to 100 nM-staurosporine, but H-7, Ro 31-7549 and Ro 31-8220 each significantly increased (by 28–38 %) vWf release above the maximum induced by thrombin alone (Table 1). In contrast, staurosporine (100 nM) decreased the vWf secretory response to histamine to 66–68 % of that induced in the absence of the inhibitor, whereas Ro 31-7549 and Ro 31-8220 had no effect on histamine-induced vWf release (Table 1).

Effect of Ca^{2+} -mobilizing agonists on PMA-induced vWf secretion

We have previously demonstrated that ATP acting at $\text{P}_{2\text{Y}}$ purinoceptors efficiently elevates $[\text{Ca}^{2+}]_i$ in human umbilical-vein endothelial cells (Carter *et al.*, 1988), though it has not previously been reported to induce vWf secretion. However, addition of $50 \mu\text{M}$ -ATP alone consistently induced a modest but statistically significant release of vWf into the culture medium (Fig. 2a).

Mean release in response to $50 \mu\text{M}$ -ATP was $24 \pm 8\%$ above unstimulated levels, and was $11 \pm 3\%$ of that induced by a maximal dose of PMA (40 nM) in the same experiment (result not shown). A lower dose of ATP ($5 \mu\text{M}$) was without effect on vWf release (Fig. 2a). When endothelial cells were challenged with a combination of $50 \mu\text{M}$ -ATP and a sub-maximal dose of PMA (16 nM), the PMA-induced response was enhanced synergistically (Fig. 2a). When $50 \mu\text{M}$ -ATP was combined with a range of PMA concentrations, a shift of the PMA dose-effect curve was observed (Fig. 2b). The secretory response induced by 80 nM-PMA was markedly elevated in the presence of ATP, and vWf release in the presence of ATP was significantly higher for each dose of PMA tested.

A comparable, though not identical, effect was obtained with the Ca^{2+} ionophore A23187 in place of ATP. A low dose of A23187 (10 nM) alone failed to induce significant release of vWf, but this sub-threshold dose of A23187 in combination with PMA (10 ng/ml) significantly enhanced release of vWf compared with the response to PMA alone (Fig. 3). A similar synergistic effect was observed with PMA and a sub-maximal dose of A23187 (100 nM; Fig. 3).

We also examined the effects of thrombin or histamine on the secretory response to a sub-maximal dose of PMA (Fig. 3), and found that either of these agonists had an additive, rather than synergistic, effect on PMA-induced secretion. However, the

Fig. 1. Effects of PK inhibitors on vWf secretion from human umbilical-vein endothelial cells induced by 40 nM-PMA

Points are means $\pm$ S.E.M. of 6–42 observations. Control release (100 %) was 15.7 ± 0.8 m-IU/ml. Key: ●, staurosporine; ▲, Ro 31-7549; ▼, Ro 31-8220.

Table 1. Effects of PK inhibitors on agonist-induced vWf secretion from human umbilical-vein endothelial cells

Results are shown as percentages of release in the absence of PK inhibitors [11.7 ± 0.3 m-IU/ml for thrombin; 23.7 ± 0.4 m-IU/ml for histamine] and are means $\pm$ S.E.M. of 6–42 observations: * significant difference ($P < 0.001$) from release in the absence of PK inhibitor.

PK inhibitor	Release (%)	
	Thrombin (2.5 units/ml)	Histamine (10 μM)
None	100 ± 2	100 ± 2
Staurosporine (100 nM)	99 ± 4	$67 \pm 2^*$
Ro 31-7549 (100 nM)	$134 \pm 5^*$	101 ± 4
Ro 31-8220 (100 nM)	$145 \pm 7^*$	105 ± 4
H-7 (50 μM)	$138 \pm 6^*$

Fig. 2. Effects of Ca^{2+} -mobilizing agonists on PMA-induced vWf secretion from human umbilical-vein endothelial cells

(a) ATP (0–50 μM) alone or with 16 nM-PMA. (b) PMA (0–80 nM) alone or with 50 μM -ATP. (c) Ionophore A23187 (0–100 nM) alone or with 16 nM-PMA. Points are means $\pm$ S.E.M. of 8–36 observations from four experiments. In (a) the asterisks indicate significant ($P < 0.001$) increases in vWf in the presence of ATP; in (c) they indicate significant ($P < 0.01$) increases in the presence of A23187. In (b) release was significantly ($P < 0.01$) greater in the presence of ATP for all concentrations of PMA.

Fig. 3. Effects of thrombin and histamine on vWf secretion from human umbilical-vein endothelial cells induced by PMA (16 nM)

Bars show means $\pm$ S.E.M. of 12 observations from four experiments. $\square$, Agonist alone; ▨ , + PMA.

maximal vWf secretory response induced by histamine (10 μM) alone was significantly enhanced in the presence of PMA.

Effects of other agonists on thrombin-induced vWf secretion

Release of vWf from endothelial cells challenged with a combination of ATP and thrombin was not significantly different from that obtained in the presence of thrombin alone (Figs. 4a and 4b), irrespective of the dose of ATP or thrombin used. Similarly, thrombin-induced vWf release was not altered in the presence of A23187 (Fig. 4c). Comparable results (not shown) were obtained with a lower concentration of thrombin (1 unit/ml).

Finally, we tested the ability of thrombin (0.6 and 1.2 units/ml) to enhance vWf release in response to sub-maximal (1 μM) or maximal (10 μM) doses of histamine, and found that it was ineffective, e.g. vWf secretion induced by 1 μM -histamine alone was 13.0 ± 0.3 m-IU/ml, and that by 1 μM -histamine plus 1.2 units of thrombin/ml was 13.4 ± 0.8 m-IU/ml (means $\pm$ S.E.M., 24 observations in two experiments).

Fig. 4. Effects of ATP or ionophore A23187 on thrombin-induced secretion of vWf from human umbilical-vein endothelial cells

(a) Thrombin (0–2 units/ml) alone or with 50 μM -ATP. (b) ATP (0–50 μM) alone or with thrombin (2 units/ml). (c) Ionophore A23187 (0–100 nM) alone or with thrombin (2 units/ml). Points are means $\pm$ S.E.M. of 9–24 observations from four experiments. Asterisks in (a) and (b) indicate significant ($P < 0.01$) release due to ATP alone.

DISCUSSION

It has previously been shown, and is confirmed in our present experiments, that either activation of protein kinase C by PMA or elevation of $[Ca^{2+}]_i$ with an ionophore induces efficient secretion of vWf from endothelial-cell storage granules (Loesberg *et al.*, 1983). However, it has not previously been investigated to what extent receptor-mediated induction of vWf secretion is mediated by either or both of these pathways, or whether other transduction pathways contribute, though Loesberg *et al.* (1983) concluded that adenylate cyclase was not involved. We have shown in preliminary experiments with electroporated human umbilical-vein endothelial cells that $[Ca^{2+}]_i$ must be elevated to supraphysiological levels by ionophore to induce vWf secretion, suggesting that this is unlikely to be the only pathway involved in vWf secretory responses to receptor stimulation (Scrutton & Pearson, 1989).

The two best-characterized agonists of vWf secretion, thrombin and histamine, each act on endothelial cells via receptors coupled to the activation of phosphoinositidase C and hence to the mobilization of intracellular Ca^{2+} (e.g. Hamilton & Sims, 1987; Hallam *et al.*, 1988). For thrombin, generation of diacylglycerol has also been monitored (Brock & Capasso, 1988). Direct measurements of PKC activation in endothelial cells, for example by translocation studies, have not been reported, but the stimulated phosphorylation of a number of proteins in intact human umbilical-vein endothelial cells by thrombin, histamine, dioctanoylglycerol and PMA has been demonstrated (Levin & Santell, 1991). Phosphorylation of one of these (P80), found in response to thrombin or dioctanoylglycerol, but apparently not to histamine, was sensitive to inhibition by staurosporine or H-7.

In the present study we have utilized novel selective inhibitors to investigate the role of PKC in vWf secretion. Staurosporine and H-7 inhibit a range of PKs, including cyclic-AMP-dependent PK, cyclic-GMP-dependent PK and Ca^{2+} /calmodulin-dependent PK, with relatively poor selectivity (Rüegg & Burgess, 1989), whereas Ro 31-7549 and Ro 31-8220, structural derivatives of staurosporine, show selectivity of at least one and often > 2 orders of magnitude in favour of PKC in a variety of assays, while retaining similar potency to staurosporine (Davis *et al.*, 1989).

Having demonstrated their potency as inhibitors of PKC-driven secretion of vWf (Fig. 1), we therefore set out to investigate their effects on secretion induced by thrombin or histamine. Unexpectedly, concentrations of Ro 31-7549 or Ro 31-8220 that virtually abolished secretion of vWf induced by PMA had no effect on histamine-induced secretion (Table 1), strongly suggesting that PKC is not involved in the stimulation of vWf secretion by histamine. Under the same conditions, staurosporine partially inhibited secretion, perhaps implicating the involvement of another PK such as that activated by Ca^{2+} /calmodulin. When thrombin was used to stimulate vWf release, none of the PK inhibitors tested was inhibitory, but the two Ro compounds and H-7 significantly enhanced release (Table 1), suggesting that for this agonist activation of PKC may decrease its ability to sustain vWf release. This could be related to feedback inhibition of coupling of the thrombin receptor, since maximal secretion requires exposure to thrombin for > 30 min (Levine *et al.*, 1982; Loesberg *et al.*, 1983). Activation of PKC with PMA uncouples Ca^{2+} -mobilizing receptors in endothelium, although for thrombin, histamine and ATP there is evidence from the use of staurosporine and H-7 against a role for PKC in the observed agonist-specific desensitization of inositol phosphate generation and Ca^{2+} release (Carter *et al.*, 1989; Halldorsson & Thorgeirsson, 1989).

Birch *et al.* (1990) have also reported data in abstract form

indicating that H-7 enhances thrombin-stimulated vWf release, and additionally that W-7 and ML-7 (inhibitors, albeit unspecific, of calmodulin and myosin light-chain kinase respectively) decreased thrombin-stimulated vWf release from intact cells and Ca^{2+} -stimulated release from permeabilized cells, implicating other kinases in the secretory response.

To investigate further whether elevation of $[Ca^{2+}]_i$ within the physiological range is involved in receptor-mediated vWf secretion, we tested the effects of ATP. Like thrombin and histamine, ATP efficiently elevates endothelial $[Ca^{2+}]_i$ and induces prostacyclin release; indeed, for thrombin and ATP there is good evidence that elevation of $[Ca^{2+}]_i$ provides the only transduction signal for prostacyclin synthesis (Hallam *et al.*, 1988; Carter *et al.*, 1988). We found, for the first time, that ATP is capable of inducing vWf secretion, but that it is much less effective than thrombin or histamine (Fig. 2). Since the ability of ATP, like that of ionophore A23187, to induce vWf release was synergistically enhanced by PMA, it is plausible that ATP is (poorly) effective when added alone, by virtue of its ability to elevate $[Ca^{2+}]_i$, but fails to be as effective as thrombin or histamine because it does not activate other necessary transduction pathways. This is consistent with the finding (Fig. 4) that neither ATP nor A23187 can increase the release of vWf induced by thrombin.

When other combinations of agonists were tested, it was found that sub-maximal doses of thrombin did not enhance release induced by histamine. However, vWf secretion induced by a sub-maximal dose of PMA was additively increased by thrombin or histamine, reaching levels above those possible in response to the receptor-mediated agonists alone (Fig. 3). This result strengthens the proposal that PKC activation is unlikely to play a major role in transducing vWf secretion in response to either of these agonists. Since maximally effective doses of thrombin or histamine alone virtually abolish the localization of vWf in granular stores in endothelial cells (Handin & Wagner, 1989), these data also raise the possibility that chronic activation of PKC with PMA alters the rate of synthesis and secretion of vWf via the constitutive pathway, and we are currently investigating this (Carew *et al.*, 1990).

We conclude that, although elevation of $[Ca^{2+}]_i$ to supraphysiological levels by the use of an ionophore can stimulate vWf secretion from endothelial cells, elevation of $[Ca^{2+}]_i$ by physiological agonists is not sufficient to induce full secretion. Further, though activation of PKC synergizes the effect of raised $[Ca^{2+}]_i$, thrombin and histamine do not use this signalling pathway to cause vWf secretion, and additional experiments are therefore needed to determine which signal-transduction mechanisms are used by these agonists.

We are grateful to the personnel of the Delivery Suite of Northwick Park Hospital for their expert help in collecting umbilical cords.

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Received 15 January 1992/19 March 1992; accepted 20 March 1992