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PHARMACOLOGICAL MODULATION OF THE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND VON WILLEBRAND FACTOR

A study in the perfused rat hindleg

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Ninette
Tranquille-Mouchabeck

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STELLINGEN

1. The acute release of t-PA does not require ongoing protein synthesis thus one can conclude that the t-PA released is derived from a stable endothelial storage pool. *This thesis.*

2. The acute release of both t-PA, a clot lysing agent and vWF, a platelet adhesion agent, is simultaneous and involves the same release inducing compounds, indicating the involvement of similar cellular pathways. *This thesis.*

3. That sodium nitroprusside prolongs the fibrinolytic activity of t-PA as observed by Korbut et al., and the observation that sodium nitroprusside reduces the acute release of t-PA from endothelial cells (*this thesis*) are not necessarily incompatible. *Korbut et al., The Lancet, 335: 669, 1990.*

4. The discrepancy relating to fibrinogen levels between the outcome of intervention studies using (fatty) fish or fish oil and of descriptive dietary surveys regarding daily fish consumption warns against drawing mechanistic conclusions from large scale population studies. *Lee et al., J. Clin. Epidemiol., 43: 913-919, 1990; Radack et al., J. Amer. Coll. Nutr., 9: 352-357, 1990.*

5. The suggestion that oxidative inactivation of PAI-1 could be physiologically important for the regulation of fibrinolysis cannot be justified on the basis of the experiments reported. *Lawrence and Loskutoff, Biochemistry, 25: 6351-6355, 1986; Strandberg et al., Fibrinolysis, 4, suppl. 3: 268, abstract, 1990.*

6. The aphysiological method to induce hyperlipaemia used by Okazaki et al. is not adequate to study hyperlipaemia related fibrinolytic changes. *Padró and Emeis, Fibrinolysis, 4: 161-167, 1990; Okazaki et al., Japan. J. Pharmacol. 52: 353-361, 1990.*

7. The general advice to the public concerning their dietary habits for the prevention of cardiovascular disease should be to just substitute liquid oil for hard fat.

8. The correlation (given by Lee et al.) between fibrinogen levels and social class is unacceptable in classifying housewives as the lowest social class. *Lee et al., J. Clin. Epidemiol., 43: 913-919, 1990.*

9. Hypercholesterolemia is not a contraindication for modern low-dose contraceptive pills.

10. A drink a day keeps the doctor away.

11. In this modern age of computers it is better to have one too many copies of a manuscript, than one too few.

12. A woman's work is never done!

Leiden, 20 February 1991

N. Tranquille-Mouchabeck

PHARMACOLOGICAL MODULATION OF THE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND VON WILLEBRAND FACTOR

A study in the perfused rat hindleg

Proefschrift

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te klokke 16.15 uur

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Ninette Tranquille-Mouchabeck

geboren te Jerusalem in 1957

"ALL IN" BV - KATWIJK

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I grow daily to honor facts more and more, and theory less and less.
Carlyle

Aan mijn supporters

Aan mijn moeder
en voor Cecil

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CHAPTER 1

INTRODUCING TISSUE-TYPE PLASMINOGEN ACTIVATOR AND VON WILLEBRAND FACTOR, AND THE MECHANISMS INVOLVED IN THEIR SECRETION FROM THE PERFUSED RAT HINDLEG

N. Tranquille

1.1 GENERAL INTRODUCTION

The vascular endothelium has recently been found to be very important in the maintenance of vascular haemostasis. Endothelial cells play a major role in the two main branches of haemostasis: fibrinolysis and coagulation. The endothelium is responsible for synthesizing fibrinolytic factors such as protein S, plasminogen activator and plasminogen activator inhibitor, and coagulation factors such as tissue factor, thrombomodulin, and von Willebrand factor. The endothelial cells also release some of these factors into the circulating blood and, moreover, provide a surface of binding sites and receptors for factors involved in both fibrinolysis such as fibrin, plasminogen activator, plasminogen, and coagulation such as Factor V, Factor X and thrombin.

The fibrinolytic activity of the blood is dependent on a large number of humoral and cellular factors, one of which is secreted into the blood from the endothelium where it is synthesized and stored. This factor is called tissue-type plasminogen activator (t-PA). Another factor which is also secreted into the blood from the endothelium where it is synthesized and stored, is von Willebrand factor (vWF). vWF is necessary for the adhesion and aggregation of platelets in coagulation. Both t-PA and vWF play important roles in haemostasis.

t-PA (reviews in Kluft, 1988) is a serine protease which activates plasminogen to plasmin in the major route of fibrin degradation. t-PA has an affinity for fibrin, and the slow activation of plasminogen by t-PA is greatly stimulated by fibrin and fibrin-related components which explain the specific thrombolytic properties of t-PA. t-PA is a complex molecule with various domains in the polypeptide structure suggesting various means for the regulation of its activity and availability. The amino-acid sequence of the mature single chain t-PA molecule has been deduced from the cDNA sequence of human melanoma t-PA (Pennica et al., 1983). t-PA consists of 527 amino-acids (with a molecular weight of 59,008 excluding carbohydrate). The t-PA molecule can be divided

into a heavy chain (275 amino-acids), and a light chain (252 amino-acids). The light chain contains the catalytic site while the heavy chain is built up of four separate domains, the finger domain, the growth factor domain and two kringle domains. t-PA can be very rapidly released into the circulation and it is also very rapidly cleared by the liver. The possible mechanisms involved in this acute release of t-PA will be discussed in more detail later.

vWF is a large glycoprotein with a complex multimeric structure. The gene for vWF encodes for a protein consisting of 2,813 amino acid residues. This protein rapidly dimerizes and undergoes a complex series of processing steps which result in the removal of a signal peptide, 22 amino acids long, a large polypeptide of 741 amino acid residues which is secreted as a distinct protein (vWFII), and the mature vWF protein subunit which consists of 2,050 amino acids (Titani et al., 1986; Bonthron et al., 1986). vWF forms part of the plasma protein factor VIII-vWF complex in plasma and has the key role in the binding of platelets to the subendothelium during vascular injury (Sakariassen et al., 1979). This is the first step in the formation of a haemostatic plug and the absence of vWF in the circulation results in a severe bleeding disorder called Von Willebrand's disease (vWD). vWD, which exists in different forms, is a result of a genetic disorder which alters either the structure and function of the vWF molecule or decreases the circulating plasma concentration of vWF (Ruggeri and Zimmerman, 1987).

1.2 ENDOTHELIAL CELLS AND SECRETION OF T-PA AND VWF

Endothelial cells are the principal, if not only, source of t-PA in the blood. Histochemical (Rijken et al., 1980) and immunohistochemical (Kristensen et al., 1984; Angles-Cano et al., 1985; Todd and Hargreaves, 1975) studies have shown that the PA activity in (vascular) endothelial cells is mainly due to t-PA. t-PA has also been found in cells of the pituitary (Kristensen et al., 1985), the pancreas, the adrenal (Kristensen et al., 1985) and in vascular smooth muscle cells (Larsson and Åstedt, 1985; Padró, personal communication).

The sub-cellular localization of t-PA has not yet been clearly established, but that endothelial cells are the major source of circulating t-PA is supported by the observation that, in the absence of blood cells, plasma and other organ systems, isolated perfused vascular beds of the kidneys, heart, lungs, ear and legs can, upon stimulation, release appreciable amounts of t-PA into the perfusate. The synthesis and secretion of t-PA by cultured bovine (Levin and Loskutoff, 1982) and human (Levin, 1983; Rijken et al., 1984; Van Hinsbergh et al., 1987) endothelial cells also confirms the association of t-PA with

endothelial cells. The rapid (acute within 1 minute) release of t-PA from cultured human umbilical vein endothelial cells in response to thrombin (Booyse et al., 1986; Kooistra and Emeis, 1988) and low density lipoproteins (Booyse et al., 1988) further supports the theory that t-PA can be released from endothelial cells.

Like t-PA, vWF is synthesized by endothelial cells (Jaffe et al., 1973,1974) and also by megakaryocytes (Nachman et al., 1977). vWF is present in the blood vessel wall in endothelial cells (Hoyer et al., 1973) and in the subendothelium (Rand et al., 1980). vWF is also stored in platelet α-granules (Nachman and Jaffe, 1975).

Unlike t-PA, the subcellular localization of vWF in endothelial cells is clearly established. The vWF is located in the Weibel-Palade bodies (Wagner et al., 1982), endothelial cell-specific organelles, which appear to be present in the endothelium of virtually all blood vessels (Weibel and Palade, 1964). Reinders et al. (1984) have described the isolation of a storage and secretion vesicle containing vWF from cultured endothelial cells, and showed that these vesicles are identical to the Weibel-Palade bodies (reviewed by Reinders et al., 1988).

Protein secretion from cells can occur in several ways. Two of the main types of secretion are constitutive secretion and regulated or induced secretion (Kelly, 1985; Gebhardt and Ruddon, 1986). Constitutive secretion occurs when newly synthesized proteins destined for secretion are rapidly transported from the endoplasmic reticulum via the Golgi apparatus to the cell surface and does not involve the use or presence of an intracellular storage compartment. Examples of constitutively secretory cells are the liver parenchymal cells, fibroblasts, and muscle cells. Induced or regulated secretion occurs as a result of a stimulus. The protein to be secreted is stored in secretory storage granules and, for a brief period, large amounts of the protein at a rate much higher than the synthetic rate, can be secreted in response to a specific stimulus. Secretion of hormones and pancreatic enzymes are examples of this type of secretion. Whether the secretion of t-PA and vWF by endothelial cells is of the constitutive or the stimulated type, is discussed below, however, more is known about the secretion of vWF than the secretion of t-PA.

vWF is secreted from endothelial cells by constitutive secretion and also by induced secretion. vWF is synthesized by endothelial cells as a large molecular weight precursor molecule. It has been implied that the vWF stored in the Weibel-Palade body for stimulatory secretion is the fully processed monomer and that this is different from the vWF that is secreted in a constitutive way, which is a co-polymer of the precursor and a fully processed subunit (Wagner and Marder, 1983,1984; Sporn et al., 1986; Mayadas

et al., 1989). The Weibel-Palade bodies secrete vWF by fusion with the plasma membrane of the endothelial cells as a result of a stimulus (McNiff and Gil, 1983).
It has also been shown that the constitutive secretion of vWF is dependent on protein synthesis (Loesberg et al., 1983), as is the case for the other adhesive proteins fibronectin and thrombospondin (Reinders et al., 1985), implying that newly synthesized protein is secreted directly. In contrast, stimulated secretion of vWF is independent of protein synthesis (Levine et al., 1982; Schorer et al., 1987) and depletes the storage pools from the Weibel-Palade bodies (and results in the disappearance of stainable particles from the cells, Loesberg et al., 1983).
t-PA baseline levels have been detected in the circulation using both antigen and activity assays (Mattsson, 1988). These levels vary between individuals but are stable for prolonged periods of time indicating an apparently stable and individually regulated level of t-PA in the blood. Endothelial cells, *in vitro,* secrete t-PA into the culture medium at a continuous and steady rate (review by Van Hinsbergh, 1988) showing constitutive secretion. However, unlike vWF, no definite storage pool of t-PA in endothelial cells has been found yet, and so a mechanism resembling induced secretion for t-PA is still not defined. That t-PA maybe surface-bound on endothelial cells and can be released from the surface receptor in response to a cellular stimulus, is a possibility which cannot be excluded. The release of t-PA into the general circulation from an extracellular storage pool (i.e. an extravascular pool in the interstitial fluid or an intravascular storage pool in the lumen of small blood vessels) also cannot be excluded. An extravascular storage pool will release t-PA due to a sudden change in the permeability of the endothelial cell lining, while luminal storage of t-PA presupposes that endothelial cells, *in vivo,* secrete t-PA constitutively into the vascular lumen and reperfusion of the vessel involved would result in a sudden discharge of the accumulated t-PA (details Emeis, 1985). However, though both these processes are theoretically possible, neither is likely to be the case for the acute release of t-PA resulting from a specific stimulus as seen in perfused vascular beds.

Release of vWF may be stimulated in cultured human endothelial cells by compounds which elevate intracellular calcium levels such as thrombin, histamine and PMA (Loesberg et al., 1983; Hamilton and Sims, 1987). The calcium ionophore, A-23187, has also been found effective in inducing secretion of vWF in human cultured endothelial cells (Loesberg et al., 1983; Reinders et al., 1985). These data suggest that stimulation of release for vWF requires calcium influx (De Groot et al,, 1984) and therefore a sustained rise in intracellular calcium and may involve the activation of protein kinase C (Newby and Henderson, 1990). This suggestion may also be valid for the secretion of t-PA. An increase in t-PA secretion from cultured human endothelial cells after stimulation with thrombin, histamine and PMA (Levin et al., 1984; Levin and Santell,

1988; Hanss and Collen, 1987) is also seen. However, as this secretion takes a minimum of 4 hours before it is detectable, this suggests an induction of the synthesis of new t-PA proteins and therefore an increase in production of t-PA, and not stimulated secretion of the t-PA from stores in the cultured cells.

Acute increases in levels of t-PA have been observed both in the circulation (Kluft et al., 1983) and in perfused vascular beds (Holemans et al., 1965; Klöcking, 1979; Kitaguchi et al., 1979; Emeis, 1983; Nakajima, 1983; Matsubara et al., 1985). The acute release of t-PA has been widely studied in different isolated perfused systems. Holemans et al. (1965) in a pioneer study showed, using the perfused dog kidney, that histamine released PA in a rapid transient manner (peaking within one minute) and that the system showed tachyphylaxis (that is to say a diminished response was seen when the stimulus was repeated with the same compound). Similar observations have been made in perfused pig or rabbit ear, dog leg, dog heart, rat heart and rat hindlegs (details Emeis, 1988). Several compounds have been found to induce the release of t-PA in these vascular systems such as acetylcholine, platelet activating factor (PAF), calcium ionophore A-23187, bradykinin, thrombin, histamine, and others (details Emeis, 1988). Most of the compounds that induce t-PA release also activate the phosphatidate-phosphoinositide cycle leading to increased intracellular calcium concentrations (Berridge et al., 1984; D'Amore and Shepro, 1977; Derian and Moskowitz, 1986; Exton, 1985; Fain and Garcia-Sainz, 1980; Farese, 1983; Rasmussen, 1986; Rubin, 1984). These compounds also activate prostacyclin production by endothelial cells probably by activating a phospholipase, and they also induce the release of endothelial cell-derived relaxing factor (EDRF). As all these endothelial release processes occur concomitantly and are induced by the same compounds, this suggests that they may all share a (partly) common pathway.

1.3 INTRACELLULAR PATHWAYS INVOLVED IN T-PA INDUCED SECRETION

Very little is known about the intracellular mechanisms involved in the induced secretion of either t-PA or vWF. The following suggestions represent the possible intracellular pathways that may be involved in the secretion of the two proteins. The hypothesis is suggested for acute t-PA release, but may also be relevant for the induced release of vWF. Figure 1 depicts diagrammatically the hypothesis which is discussed in more detail below.

It is assumed that t-PA is released from endothelial stores by the interaction of a specific ligand or agonist with its endothelial cell receptor leading to the activation of the phosphatidate-phosphoinositide pathway, increased intracellular calcium concentrations

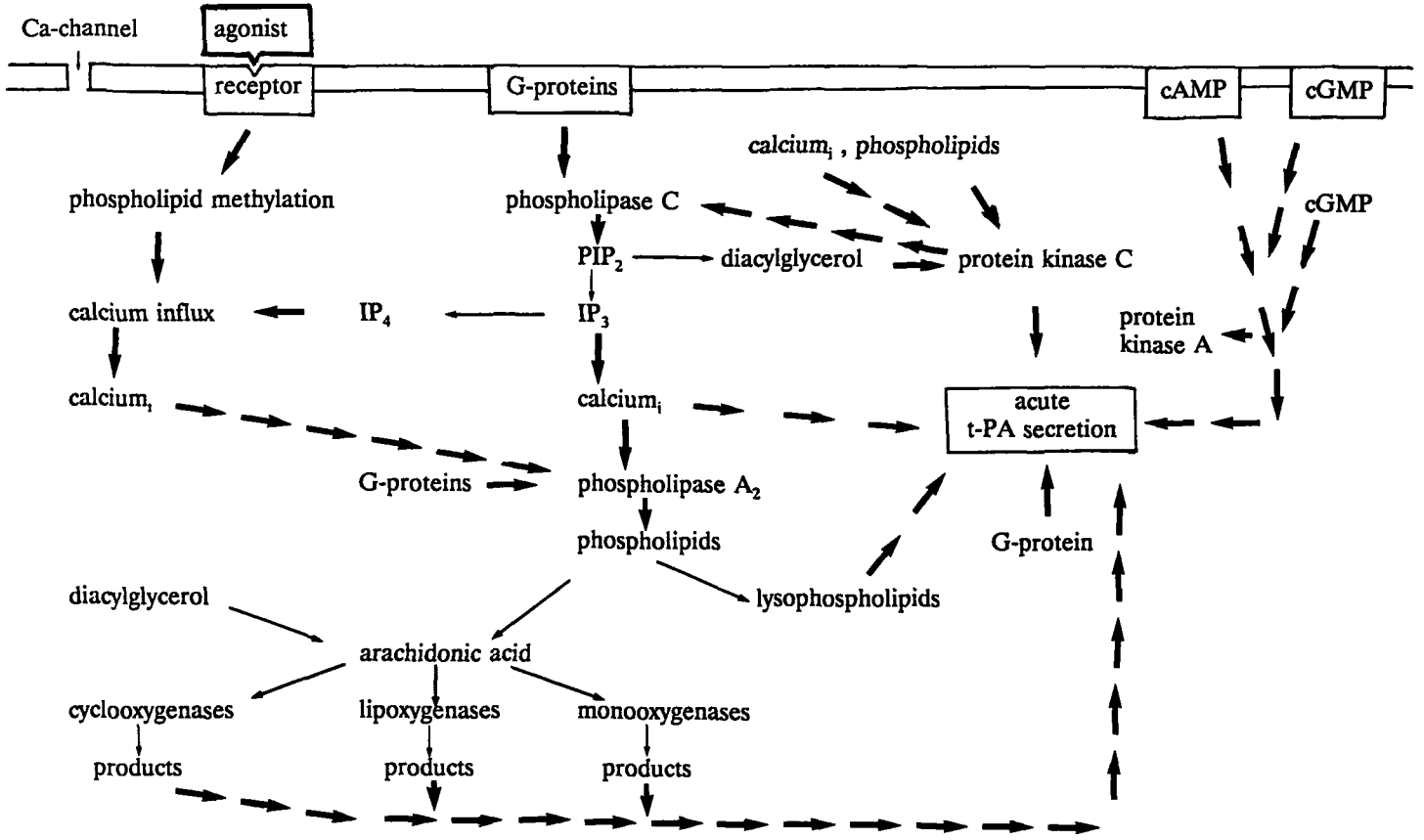

Figure 1. Working hypothesis of the possible cellular mechanisms involved following stimulation to induce t-PA (or vWF) secretion.
Abbreviations: PIP_2 - phosphatidylinositol 4-5-biphosphate; IP_3 - inositol 1,4,5-triphosphate; IP_4 - inositol 1,3,4,5-tetrakisphosphate; calcium$_i$ - intracellular calcium.
Arrows: ⟶ product formation; ➡ effect: either activation or inhibition.

and calcium influx, enzyme activation and formation of arachidonic acid metabolites, finally resulting in the induction of the release reaction.

About a century ago Paul Ehrlich proposed the idea that cells possess on their surfaces defined chemical entities which act as recognition sites for other molecules. He based this on a study of immune specificity and it is now the basis of the accepted drug receptor theory. The binding of an agonist to its receptor is the first step in the reaction and the next chemical signal generated in response to this binding is the 'second messenger' in the process and sets in motion the intracellular responses to that reaction. This may involve a change in the activity of an enzyme (e.g. adenylate cyclase; phospholipase C), or the opening of an ion channel or a change in the conformation of an intermediate protein as a signal transducer to amplify the response. The processes are very complex, and different agonists having separate, specific receptors can utilize similar intracellular systems in their target cells. Many cell surface receptors exert their actions through specific guanine-nucleotide binding of regulatory proteins, called the 'G'-proteins. There is evidence now that a family of these signal transducing G-proteins exists, each formed of a GTP-binding α-subunit, a β-subunit, and an γ-subunit (Birnbaumer, 1990).

These G-proteins act as go-betweens, coupling receptors to the appropriate signal generator system on the plasma membrane of the cell by exchanging GTP for the GDP-bound to the α-subunit of the G-protein in question (Gilman, 1987). The distinct α-subunits derived from the different G-proteins transduce signals for a variety of effector systems such as stimulation and inhibition of adenylate cyclase activity, regulation of calcium and potassium ion channels and stimulation of inositol phospholipid metabolites, to name a few (Houslay, 1987; Limbird, 1988; Axelrod et al., 1988; Birnbaumer, 1990). Bacterial toxins have been found to be very selective in the identification and regulation of the different G-proteins (Gilman, 1987; Neer and Clapham, 1988). Cholera toxin has been found to regulate the activity of G_s - a G-protein which mediates stimulation of adenylate cyclase activity (Jacquemin et al., 1986). Pertussis toxin is also used in G-protein identification (Reisine, 1990), and catalyses the ADP-ribosylation of G_i, an inhibitory G-protein (Bokoch et al., 1983; Itoh et al., 1986). The α-subunit of G_i is a good substrate for pertussis toxin, however, a number of other G-proteins are also substrates for pertussis toxin such as G_o, a protein found in high quantities in the brain (Neer and Wolf, 1984; Sternweiss and Robishow, 1984) which mediates regulation of different ionic conductance channels. Flavahan et al. (1989) studied the effects of pertussis toxin on the endothelium and endothelium-dependent and -independent relaxation in porcine coronary arteries. They demonstrated that pertussis toxin does interfere with the release of EDRF's stimulated by certain endothelial activators only, and concluded that the release of EDRF's may occur through different pathways some

of which involve a pertussis-sensitive G_i-protein-dependent mechanism. Pirotton et al. (1987) showed that pertussis toxin and cholera toxin enhanced the stimulated release of prostacyclin from bovine aortic endothelial cells. Their results suggest that a pertussis-sensitive GTP-binding protein is involved and may play a role in the control of prostacyclin biosynthesis. The above data suggest that G-proteins may be involved in the process that leads to the stimulated acute release of t-PA or vWF from endothelial cells.

The possible involvement of the cyclic nucleotides, cyclic AMP (cAMP) and cyclic GMP (cGMP), in the secretion of t-PA or vWF should also be considered. The importance of cAMP as a second messenger involved in the mediation of agonist-receptor responses was recognized since its discovery in the late 1950's (Sutherland, 1971). Most cells possess a plasma membrane-assimilated enzyme, adenylate cyclase, which can produce cAMP from ATP so that an extracellular signal can be transduced across the plasma membrane to produce an intracellular response. However, in the early 1970's it was discovered that GTP (coupled to G-proteins) was necessary for the activation of adenylate cyclase. Guanylate cyclase was also identified and found to exist in two distinct forms, one membrane-bound and the other soluble. The membrane-bound or particulate form of the enzyme is likely to act as a transducer of extracellular signals in cells. In the early 1960's when cGMP was first discovered (Hardman, 1971), cGMP was also thought to act as an intracellular second messenger, similar to cAMP. Endothelial cells contain guanylate cyclase in both its soluble and particulate form. The soluble guanylate cyclase is activated by nitric oxide and sodium nitroprusside, and the particulate cyclase is activated by atriopeptins (Adams Brotherton, 1986; Martin et al., 1988). Some of the compounds that induce the release of t-PA and vWF such as thrombin, bradykinin and histamine all elevate cGMP levels in the endothelium (Adams Brotherton, 1986). Endothelial cells are known to contain an adenylate cyclase that can be activated by forskolin, β-adrenergic agonists, prostaglandin E and prostacyclin (e.g. Adams Brotherton and Hoak, 1982; Karnushina et al., 1983; Whorton et al.,1982). With the above information provided, the possible involvement of the cyclic nucleotides in the intracellular mechanisms of t-PA and vWF secretions cannot be ignored.

One component that is widely involved in different intracellular messenger systems is calcium. The role of calcium in various cellular secretory processes has been firmly established (review Rubin, 1984). Whether calcium is a major mediator of t-PA or vWF stimulus induced secretion from endothelial cells should be anticipated especially as Lückhoff (1988) and Adams et al. (1989) have recently reviewed the evidence that calcium is a second messenger for EDRF and prostacyclin release from endothelial cells. The binding of a drug onto its membrane surface receptor can be coupled to an elevation in cytosolic calcium levels, which can be due either to calcium entry via ion

channels in the plasma membrane or to calcium release from intracellular stores (review Rasmussen, 1990), or both. Newby and Henderson (1990) suggest that intracellular calcium appears to mediate the acute stimulation of endothelial secretion whether it occurs by synthesis from a precursor (as for prostaglandins and EDRF) or by release from a stored pool (as for vWF from the Weibel-Palade bodies). However, the difference in secretion may involve different intracellular calcium pools or the involvement of another second messenger system such as protein kinase C.

Another aspect of this discussion involves the membrane phosphoinositides. A large number of different agonists can stimulate an increase in the metabolism of membrane phosphoinositides. The receptors involved are multifunctional in nature and have been implicated as part of a general transducing mechanism for the activation of phospholipase C, the mobilization of calcium, the activation of protein kinase C, the release of arachidonic acid and the activation of guanylate cyclase to form cGMP (Berridge, 1981). The main reaction of this transducing mechanism is the hydrolysis of a specific membrane phosphoinositide which produces two products, diacylglycerol and inositol tri-phosphate, both of which may function as second messenger. Diacylglycerol stimulates a specific Ca^{++} -dependent protein kinase, protein kinase C (Nishizuka, 1984a,b) and inositol tri-phosphate mobilizes intracellular calcium (Michell, 1975; Berridge, 1987). These two parts of the inositol lipid messenger system can function independently to regulate cell activation and they can also act synergistically (under some circumstances) to induced heightened cellular responses.

Changes in protein phosphorylation affects the regulation of many diverse aspects of cell function. One of the protein kinases that respond to different signals in cells, protein kinase C, alters the phosphorylation state of cellular proteins on specific serine or threonine residues and is believed to have a major role in cellular regulation. There are at least seven different forms of protein kinase C distributed in the various cell tissues and organs of the body (Nishizuka, 1988). Most cell types contain more than one species of protein kinase C and different cell functions may involve different protein kinase C-species. Protein kinase C is only physiologically active when bound to membranes and its activity is dependent on the nature of its lipid environment (Epand and Lester, 1990). The possible functions of protein kinase C include its involvement in modulation of ion conductance, regulation of receptor interaction in signal transduction, smooth muscle contraction, gene expression, cell proliferation, secretion and exocytosis (Nishizuka, 1986). It is this possible involvement of protein kinase C in cellular secretion mechanisms, such as t-PA, that is of interest in this discussion. The involvement of protein kinase C in the release of EDRF and prostacyclin has been established from the actions of phorbol esters in cultured endothelial cells (De Nucci et al., 1988; Demolle and Boeynaems, 1988; Demolle et al., 1988; Lewis and Henderson, 1987).

The involvement of arachidonic acid, acting as a second messenger, in the secretory process of t-PA (and vWF) must also be considered. Arachidonic acid may be derived from phospholipids or diacylglycerol, and can be metabolized by different routes to yield a very large number of pharmacologically distinct products referred to as eicosanoids. The main pathways of arachidonic acid metabolism found in animal tissues are the cyclo-oxygenase, lipoxygenase and cytochrome P-450 mono-oxygenase pathways as shown in Figure 1. The cyclooxygenase pathway leads to the formation of prostaglandins, prostacyclin and thromboxane A_2. The lipoxygenase pathways leads to the formation of the HETES and leukotrienes, and cytochrome P-450 catalyses the conversion of arachidonic into an array of epoxyeicosatrienoic acids. Two possible intracellular mechanisms have been suggested which may contribute to a direct effect of arachidonic acid itself in cells. The first of these involves the activation of one or more protein kinase enzymes, especially protein kinase C (McPhail et al., 1984), and the second involves the release of calcium from intracellular storage sites (Wolf et al., 1986). The intracellular actions of arachidonic acid and its lipoxygenase metabolites (including the regulation of membrane ion channels and protein kinases), have led Piomelli and Greengard (1990) to suggest that these lipophilic molecules may regulate or be involved in the modulation of neurotransmitter release. This mechanism, suggesting arachidonic acid and its metabolites may act as intracellular second messengers, leads to their possible involvement in t-PA or vWF release from endothelial cells.
It can be deduced from the above summary of the potential intracellular pathways, that the specific intracellular pathway involved in the acute secretion of t-PA (and vWF) may involve and depend on several different intracellular routes and second messenger systems (as seen in Figure 1).

1.4 THE AIM OF THIS STUDY

The aim of these studies described in this thesis was to further elucidate intracellular mechanisms and pathways involved in the acute release of t-PA and vWF from the vascular endothelial cells as a result of a specific stimulus. These deal with the acute release of t-PA and vWF from the perfused rat hindleg and investigate the possible pathways involved as shown in Figure 1.

The experimental model developed by Emeis (1983) of the perfused rat hindleg region, provides one with a physiological perfusion system covering a large surface area of blood vessel walls (the lower abdomen, legs and tail of the animal) to work with (see Figure 2). Being an *ex vivo* perfusion model one does not have the interference of vaso-active, fibrinolytic or coagulating substances released from the circulating blood, cells, and

platelets, and so one is only in contact with the endothelial cells of the vascular wall. The rat as an experimental model is far more acceptable for use than the dog or rabbit, especially when such a large study is undertaken. The isolated pig ear is also a useful model, however, the time delay in actually obtaining the ears before commencing the perfusion (about 3 hours), and the very slow rate of perfusion compared to the physiological flow rate used in the rat hindleg model suggest that the rat hindleg model is a more reliable experimental model to use. Another reason for choosing the rat as an experimental model is the following: the fibrinolytic system of the rat is very similar to that of a human, and also rat t-PA closely resembles human t-PA.

The first issue we investigated was whether the acute release of t-PA was due to constitutive or stimulated secretion, or in other words, whether the t-PA was synthesized and immediately released in response to the stimulus or whether it was released from stable endothelial stores of t-PA in response to the stimulus. To try and establish the type of secretion involved in the acute release of t-PA from the perfused rat hindleg, we studied the effects of protein synthesis inhibition by cycloheximide pretreatment from 1 to 5 hours (chapter 2).

Early studies by Emeis and Kluft (1985) had shown that the acute release of t-PA from the perfused rat hindleg is inhibited by the lipoxygenase inhibitors nor-dihydroguaiaretic acid or AA-861, and by the leukotriene synthesis inhibitor diethyl carbamazine. This suggested that the release of t-PA may be dependent on the lipoxygenase pathway of arachidonic acid metabolism and this led to the series of experiments involving the leukotrienes and prostaglandins reported in chapter 3.

In humans both t-PA and vWF are simultaneously released into the blood by various stimuli, such as venous occlusion, exercise and infusion of adrenaline and 1-desamino-8-D-arginine vasopressin (DDAVP) (Cash et al., 1974; Mannucci et al., 1975; Marsh and Gaffney, 1980; Nilsson et al., 1982; Prowse et al., 1984). In chapter 4 we have investigated whether in our experimental model of the perfused rat hindleg the induction of acute release of t-PA was also accompanied by release of vWF.

A broader look at the involvement of the phospholipase pathway and eicosanoid metabolism in the acute release of t-PA from rat endothelial cells was the next intracellular pathway to be investigated in chapter 5.

Continuing the study of intracellular mechanisms that may be involved in the acute release of t-PA and vWF, the role of calcium was investigated in our experimental model (chapter 6).

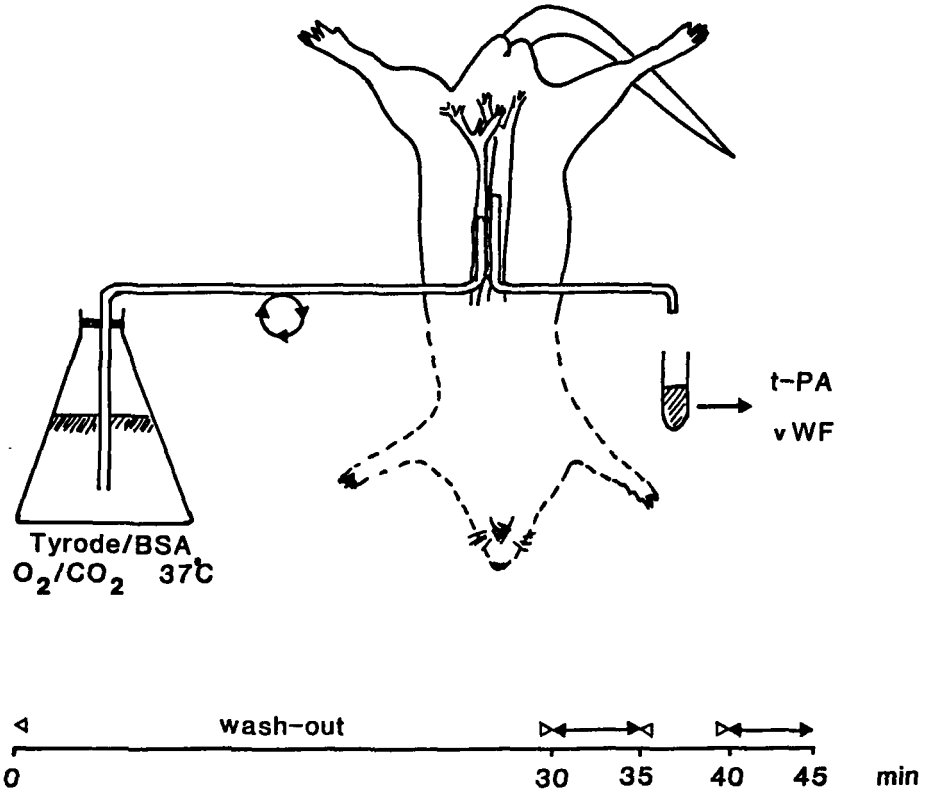

Figure 2. Diagrammatical representation of the rat hindleg perfusion system.

The involvement of cAMP and cGMP in the synthesis and release of prostacyclin and EDRF, respectively, from cultured endothelial cells led to the study conducted in chapter 7. The involvement of the cyclic nucleotides, as second messengers in the acute release of t-PA and vWF from intact vascular endothelial cells was the subject which was studied.

This led to the final study presented in this thesis, chapter 8, which involved a clinically used drug, pentoxifylline, thought to have fibrinolytic activity. The mechanism of action of this drug and its first metabolite was thought to be similar to that of a cyclic nucleotide phohsphodiesterase inhibitor. These compounds were investigated using our hindleg perfusion model.

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CHAPTER 2

PROTEIN SYNTHESIS INHIBITION BY CYCLOHEXIMIDE DOES NOT AFFECT THE ACUTE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

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SUMMARY

The acute release of tissue-type plasminogen activator (t-PA) was studied in perfused rat hindlegs. Pretreatment of rats with the protein synthesis inhibitor cycloheximide (2 mg/kg) at 1, 3 or 5 h prior to perfusion of rat hindlegs did not influence the amount of t-PA released by platelet-activating factor (20 nM) or bradykinin (1 μM). The amount of t-PA activity that could be extracted from hindleg skeletal muscle was not decreased by cycloheximide pretreatment though it was decreased in lung extracts. The *in vivo* release of t-PA was not affected by cycloheximide pretreatment. The data suggest that the acute release of t-PA from vascular endothelial cells does not require ongoing protein synthesis, but that acutely released t-PA is derived from a stable endothelial storage pool.

INTRODUCTION

Vascular endothelial cells *in vivo* contain tissue-type plasminogen activator (t-PA) as shown by histochemical and immunohistochemical techniques (1-3), and will respond to certain stimuli by the rapid release of t-PA into the circulation (4-6). Using perfused rat hindlegs as a model system to study the t-PA release reaction, we have shown previously that following stimulation by e.g. platelet-activating factor (PAF), bradykinin, thrombin or leukotrienes the t-PA content of rat hindleg perfusates will increase rapidly (7-9). Peak levels of t-PA are found within 1 minute after adding release-inducing compounds to the perfusion fluid. Cells can secrete proteins by two pathways: by constitutive secretion, which requires ongoing protein synthesis, or by induced secretion from a

cellular storage pool (see e.g. ref. 6, and the discussion section). To decide whether the t-PA released from perfused hindlegs was derived from stores of t-PA, or from recently-synthesized t-PA, we studied the release of t-PA after inhibiting protein synthesis by cycloheximide for periods of 1-5 h. The effect of protein synthesis inhibition by cycloheximide on the t-PA content of hindleg skeletal muscle and lung was studied as well. The results showed that continuing protein synthesis was not required for t-PA release to occur, and that tissue levels of t-PA were little affected by prolonged inhibition of protein synthesis. We suggest that the t-PA released from endothelial cells following stimulation is derived from a stable endothelial storage pool of t-PA.

MATERIALS AND METHODS

Methods.

Male Wistar rats (Broekman Institute, Helmond, The Netherlands) weighing 220-330 g, anaesthetized using pentobarbitone (Nembutal; 60 mg/kg intraperitoneally) were used in all the following experiments.

Rat hindlegs perfusion.

The release of tissue-type plasminogen activator (t-PA) from a perfused vascular bed was studied using the perfused rat hindleg system as described previously (7,9). In brief: the rat hindleg region was perfused, using a roller-pump at constant flow (9-10 ml/min), through the aorta with oxygenated Tyrode's salt solution (composition in mM: NaCl 146, KCl 6, $CaCl_2$ 3, $MgCl_2$ 0.5, KH_2PO_4 0.3, $NaHCO_3$ 20, glucose 5.6) containing 0.1 mg/ml bovine serum albumin (BSA), pH 7.4 at 37°C. Perfusate samples were collected from an outflow cannula inserted into the inferior vena cava.

To clear the vessels from residual blood, each experiment was started with a 40 min perfusion of the Tyrode/BSA solution. The compound used as t-PA release stimulus (either platelet-activating factor (PAF) or bradykinin) was then added to the Tyrode/BSA solution and perfused through the hindleg region for 5 min. A further 5 min wash-out period with the Tyrode/BSA solution was carried out before a second stimulus (PAF, when bradykinin had been used as the first stimulus, or vice versa) was applied. Perfusate samples were collected every 30 sec for 30 sec, kept on ice till the experiment was completed and then centrifuged (3,000 x g for 10 min). The supernatant was collected and mixed at a 10:1 ratio with a solution containing 0.5 M Tris. HCl (pH 7.5) and 1% Triton X-100 and assayed immediately, or the samples were stored at -20°C.

Cycloheximide treatment.

Test animals were preinjected with cycloheximide (2 mg/kg body weight i.v.) (10,11) at

1 h, 3 h or 5 h before perfusion. The 5-h test animals received a second injection of cycloheximide (2 mg/kg) at 2½ h before the perfusion was carried out. Cycloheximide-treated animals not used for experiments survived and behaved normally for several weeks, apart from slight diarrhea during the first 24 h. Control animals were injected with saline (1 ml/kg). The cycloheximide pretreated animals were initially perfused for 10 min with Tyrode/BSA solution and then for a further 30 min with Tyrode/BSA solution containing cycloheximide (2 μg/ml) before being stimulated with PAF or bradykinin.

Effect of cycloheximide on tissular protein synthesis.
Animals were i.v. injected with cycloheximide (2 mg/kg) or saline. At 2 h 40 min the rats were reanaesthetized and injected with ^{35}S-methionine (80 μCi/kg). Twenty min later 1-gram pieces of liver, lung and hindleg skeletal muscle were obtained. Trichloroacetic acid-insoluble radioactivity in these tissues was determined as described by Farber and Farmar (10), except that finely-minced tissues were homogenized using a Polytron PTA-7 (Kinematica GmbH, Littau-Luzern, Switzerland).

Spectrophotometric plasminogen activator (PA) activity assay.
The PA activity of samples was determined by the indirect spectrophotometric rate assay described by Verheijen et al. (12). Details are given in Tranquille and Emeis (9). Sample volumes were 30 μl for the perfusate samples and 2-10 μl for the tissue extracts.
Dilutions of human melanoma t-PA (13) were run in each plate for calibration. The PA activity of the samples will be expressed in International Units (IU), as defined by the International Standard of t-PA (14). t-PA antigen could not be determined in the perfusate samples as no antigen assay for rat t-PA is presently available.

Quenching of PA activity.
The quenching experiments used the same spectrophotometric assay as mentioned above with the following additions: to the buffer, fibrin digest, and sample, 0-40 μl of a rabbit anti-human t-PA IgG solution or amiloride (final concentration 25-100 μM) was added. The plate was incubated for 10 min at 37°C and then the substrate S-2251 and plasminogen were added and the incubation started.

***In vivo* release of PA.**
Rats were injected i.v. with cycloheximide at a dosage of 2 mg/kg 3 h before experimentation. Controls received saline (1 ml/kg). Blood samples were obtained from a cannula in the carotid artery before and at 1, 2, 3, 5, 7 and 10 min after injection of PAF (1 μg/kg) or bradykinin (50 μg/kg). Blood (0.2 ml) was diluted to 10% in 1.7 ml of 0.12 M sodium acetate (pH 7.4), clotted with 0.1 ml thrombin (20 NIH U/ml) and

incubated at 37°C. Lysis times were read in min. When indicated, antibodies were added to the diluted blood 5 min before the addition of thrombin.

Fibrin autography.

Sodium dodecyl sulphate/8% polyacrylamide slab gels were prepared according to Laemmli (15). Fibrin autography was performed according to Granelli-Piperno and Reich (16), as described in detail elsewhere (17).

Assay of t-PA in muscle and lung tissue extracts.

Tissue extracts were analyzed for their t-PA content as follows. Lungs were removed from the rats, just after the perfusion was started (and just before the pulmonary arteries were severed) and immediately frozen at -20°C. The biceps femoris muscles were removed from the thigh of the rat before perfusion or after the perfusion was completed (see Table 3) and also stored frozen.

On thawing each tissue was rinsed with phosphate-buffered saline, dried on blotting paper, weighed and very finely minced. The tissue sample was then suspended (1 g wet weight per 5 ml) at 4°C in a slightly modified variation of Camiolo's buffer (18) (composition in mM: CH_3COOH 75, NaCl 225, KCl 75, EDTA 10, arginine 100, 0.25% Triton X-100, pH 4.2 adjusted using 1 N HCl) and homogenized using a Polytron PTA-7 at maximum speed for 90-120 sec at 4°C. After homogenization, the sample was frozen using liquid nitrogen and thawed at 37°C three times to increase the t-PA activity extracted. The sample mixture was then centrifuged at 3,000 x g for 15 min at 4°C, and the supernatant assayed immediately for PA activity. The lung tissue samples were treated similarly to the muscles, however, they were centrifuged immediately after homogenization and the supernatant assayed for activity. The protein content of the tissue extracts was determined according to Lowry's method (19) using BSA as a standard.

Materials.

Platelet-activating factor was obtained from Bachem (Bubendorf, Switzerland). Nembutal was from Sanofi (Paris, France). Bovine thrombin from Leo Pharmaceuticals (Ballerup, Denmark). Bradykinin, bovine serum albumin, cycloheximide and amiloride were from Sigma (St. Louis, U.S.A.). Purified human t-PA (two-chain) was a gift from Dr. J. H. Verheijen of the Gaubius Institute. Rabbit anti-human t-PA IgG was from Cooper-Organon Teknika (Turnhout, Belgium). L-[^{35}S]-methionine (1,151 Ci/mmole) was from NEN Research Products (Den Bosch, The Netherlands).

The materials used in the spectrophotometric PA assay have been detailed elsewhere (12). All other materials were of analytical grade.

RESULTS

The effect of cycloheximide on protein synthesis.

Pretreatment of rats with cycloheximide for 3 h reduced the incorporation of radiolabelled methionine into trichloroacetic acid-insoluble protein by 90 ± 4% (mean ± sd; n = 3) in liver, by 82 ± 4% in lung and by 92 ± 3% in hindleg skeletal muscle, compared to the incorporation in saline-injected controls (n = 3). The chosen cycloheximide treatment schedule was thus sufficient to adequately inhibit protein synthesis during the experimental periods.

The effect of cycloheximide on t-PA release from perfused rat hindlegs.

The effect of cycloheximide on the release of t-PA was studied in the hindleg perfusion experimental model. The experimental animals were pretreated with cycloheximide (2 mg/kg) at timed intervals of 1 h, 3 h or 5 and 2½ h before the start of the perfusion. PAF (20 nM) or bradykinin (1 μM) were used as stimulators to induce release of t-PA from the hindleg. The two compounds were used in each experiment either as the first stimulus or as the second. When PAF was used as the first stimulus, pretreatment with cycloheximide had no effect on the amounts of t-PA released (Table 1). The time course of t-PA release during the five min stimulation period was also identical in the control and cycloheximide-treated rats. However, used as the second stimulus after bradykinin, the amount of t-PA released by PAF was significantly reduced by 42% after 3 h, and by 49% after 5 h of cycloheximide pretreatment, though not after 1 h (Table 1).

Table 1. Effect of pretreatment with cycloheximide (2 mg/kg) on PAF-induced release of t-PA from perfused rat hindlegs.

Pretreatment	PAF (20 nM) as first stimulus		PAF (20 nM) as second stimulus	
	t-PA released (IU/ml)	Mean flow (ml/min)	t-PA released (IU/ml)	Mean flow (ml/min)
No cycloheximide pretreatment	3.39 ± 0.08 (4)[1]	9.5	3.07 ± 1.05 (5)	8
Cycloheximide (-1 h)	n.d.[2]	-	2.73 ± 0.30 (4)	5
Cycloheximide (-3 h)	3.97 ± 0.47 (4)	11	1.79 ± 0.41 (4)[3]	3.5
Cycloheximide (-5 h and -2.5 h)	3.92 ± 0.35 (4)	10	1.58 ± 0.34 (4)[3]	3.0

[1] Mean ± s.d. (number of experiments in parentheses).

[2] n.d.: not done.

[3] Significantly different from no cycloheximide control ($p < 0.01$) by analysis of variance, followed by Bonferroni's modified t-test (29).

Similar effects were noted when bradykinin was used as the stimulus to induce t-PA release: no effect of cycloheximide on t-PA release was observed when bradykinin was used as the first stimulus, but when bradykinin was used as the second stimulus after PAF the amount of t-PA released was decreased after cycloheximide pretreatment for 5 h, though not after 3 h (Table 2). Of note was that in control animals bradykinin released significantly less t-PA when used as the second stimulus after PAF (Table 2).

Table 2. Effect of pretreatment with cycloheximide (2 mg/kg) on bradykinin-induced release of t-PA from perfused rat hindlegs.

Pretreatment	Bradykinin (1 μM) as first stimulus		Bradykinin (1 μM) as second stimulus	
	t-PA released (IU/ml)	Mean flow (ml/min)	t-PA released (IU/ml)	Mean flow (ml/min)
No cycloheximide pretreatment	1.62 ± 0.67 (5)[1]	10	0.71 ± 0.04 (4)[3]	3.4
Cycloheximide (-1 h)	1.66 ± 0.12 (4)	9	n.d.	-
Cycloheximide (-3 h)	1.39 ± 0.12 (4)	9	0.74 ± 0.16 (4)	3.0
Cycloheximide (-5 h and -2.5 h)	2.13 ± 0.46 (4)	10	0.43 ± 0.09 (4)[4]	1.3

[1] Mean ± s.d. (number of experiments in parentheses).

[2] n.d.: not done.

[3] Significantly different from bradykinin as first stimulus ($p < 0.05$) by Student's t-test (29).

[4] Significantly different from no cycloheximide controls ($p < 0.01$) by analysis of variance, followed by Bonferroni's modified t-test (29).

Bradykinin and PAF induced no detectable oedema formation (as judged visually and by the absence of changes in perfusion pressure and flow) when applied as first stimuli. However, during the second stimulation period oedema formation was observed visually, and the flow of perfusate decreased, most pronounced during perfusion with bradykinin after PAF had been applied as the first stimulus (Table 1 and 2). The decrease in perfusate flow was more severe in animals pretreated with cycloheximide. In cases of reduced flow an increase of perfusion pressure of about 1 cm Hg was also observed.
When, for the various experimental groups, the mean flow was plotted against the mean amount of t-PA released (Fig. 1; data from Tables 1 and 2), a positive correlation between flow and t-PA release was found for both PAF-induced release ($r = 0.972$, $n = 7$, $p < 0.01$) and bradykinin-induced release ($r = 0.954$, $n = 7$, $p < 0.01$).
No t-PA was released by perfusion of rat hindlegs with only Tyrode/BSA, with or without pretreatment with cycloheximide. The PA activity released by PAF or bradykinin

was plasminogen-dependent and fibrin-dependent, and could be fully quenched by anti-human t-PA IgG, in agreement with previous studies (8,9).

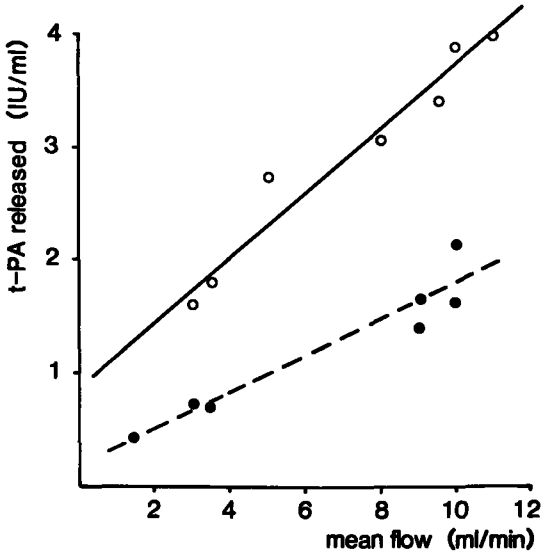


Fig. 1. Correlation between mean flow (ml/min) and the concentration of t-PA in the perfusate after stimulation with 20 nM PAF (o) or with 1 μM bradykinin (•). Data are replotted from Tables 1 and 2. Correlation coefficients are +0.972 for PAF-induced release and +0.954 for bradykinin-induced release (both: $p < 0.01$).

The effect of cycloheximide on *in vivo* t-PA release.

Rats were injected with cycloheximide (2 mg/kg i.v.) or with saline, 3 h before the injection of PAF (1 μg/kg i.v.). Both in the control and in the cycloheximide-pretreated animals the injection of PAF rapidly resulted in large reductions of the dilute blood clot lysis times, to the same extent (Fig. 2a). The only difference between the two groups was that in the cycloheximide-injected animals the clots prepared from blood taken before the injection of PAF did not lyse within 24 h; in the control group the lysis time of these clots was 110 ± 10 min (mean ± sd; n = 4). Similar results were obtained when bradykinin (50 μg/kg) was used to induce t-PA release (data not shown). The increased blood fibrinolytic activity could be quenched by preincubation of the diluted blood with anti-human t-PA IgG (Fig. 2b).

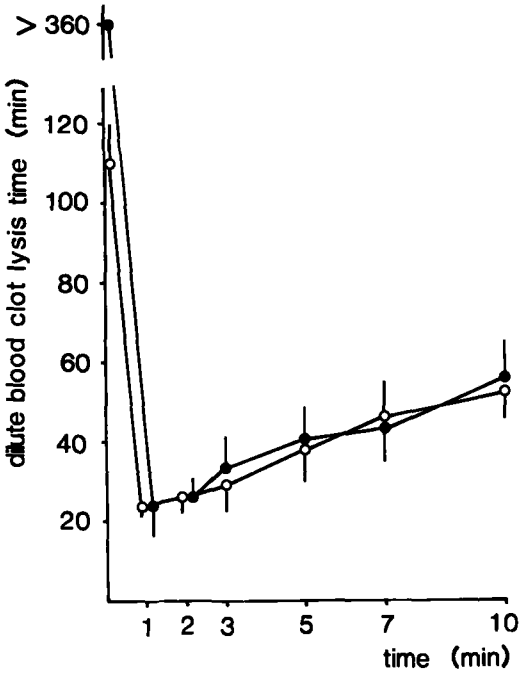


Fig. 2. a) Time course of the effect of PAF (1 μg/kg i.v.) on the dilute blood clot lysis time in control rats (o) and in rats pretreated with cycloheximide (2 mg/kg i.v.) three h before PAF (•). Data are shown as mean lysis time from four rats; vertical lines indicate s.d.

The effect of cycloheximide on the t-PA content of tissue extracts.

Tissue extracts were initially prepared exactly as described by Camiolo et al. (18). However, the buffer system used by these authors seriously interfered with our spectrophotometric PA assay, resulting in non-linear dose-response curves when increasing sample volumes of tissue extract were assayed. None of the components of the buffer system by itself influenced the activity of human t-PA standards when added separately. By adjusting the pH of the buffer to 4.2 with 1 N HCl instead of the prescribed acetic acid, a modified buffer was obtained (see Methods) which reduced the activity of t-PA standards only slightly in the spectrophotometric assay used (1% for each μl of modified buffer added to the standard t-PA assay). Therefore, a maximal sample

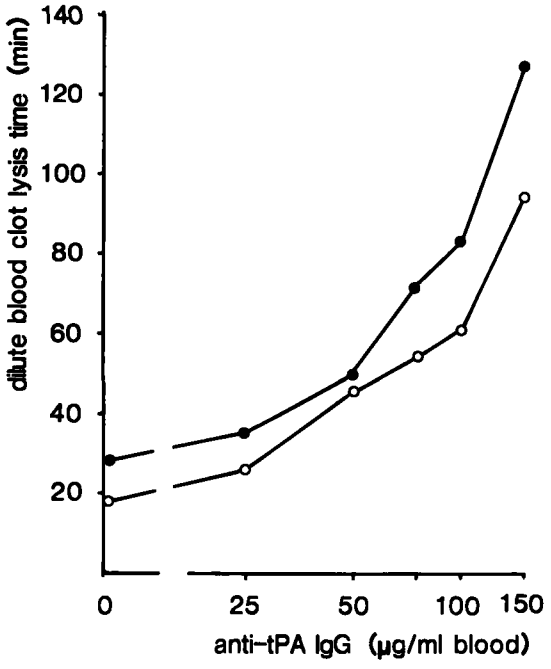


Fig. 2. b) Effect of preincubation with anti-human t-PA IgG on the dilute blood clot lysis time of blood samples obtained from two cycloheximide-pretreated rats (2 mg/kg; 3 h). Blood was obtained one minute after the injection of PAF (o) or bradykinin (•) (one rat for each compound). For details, see Materials and Methods.

volume of 10 µl was chosen, maintaining at least 90% of the sample activity. Tissue extracts were assayed immediately after extraction, as the PA activity was found to be reduced on storage.

Pretreatment with cycloheximide had no significant effect on the amount of PA activity (expressed as IU/mg protein extracted), found in the muscle extracts. However, in the lung extract the amount of PA activity was reduced by 41% after pretreatment with cycloheximide for 5 h. ($p < 0.05$). The amount of protein in the muscle extracts was found to be slightly lower after pretreatment with cycloheximide (Table 3). In extracts t-PA antigen concentrations were not determined, as no antigen assay for rat t-PA

antigen is available. No PA inhibitor activity was found in any tissue extract by the method of Verheijen (20).

Table 3. **Plasminogen activator activity in tissue extracts from hindleg muscle and lung.**

Tissue source	PA activity (IU/g wet weight)	Protein extracted (mg/g wet weight)	PA activity (IU/mg protein extracted)
Nonperfused control muscle (n = 6)	4.66 ± 0.92[1]	21.5 ± 6.0	0.22 ± 0.07
Control muscle after perfusion (n = 4)	3.94 ± 1.45	21.0 ± 4.0	0.19 ± 0.08
Cycloheximide-treated muscle (-3 h) after perfusion (n = 8)	3.41 ± 0.92	18.0 ± 3.0	0.19 ± 0.06
Cycloheximide-treated muscle (-2.5 h and -5 h) after perfusion (n = 8)	3.07 ± 0.42[2]	15.5 ± 2.0[2]	0.20 ± 0.04
Control lung (n = 4)	559 ± 113	45.5 ± 7.5	12.29 ± 3.20
Cycloheximide-treated lung (-2.5 h and -5 h) (n = 4)	375 ± 105	52.0 ± 8.0	7.21 ± 2.29[3]

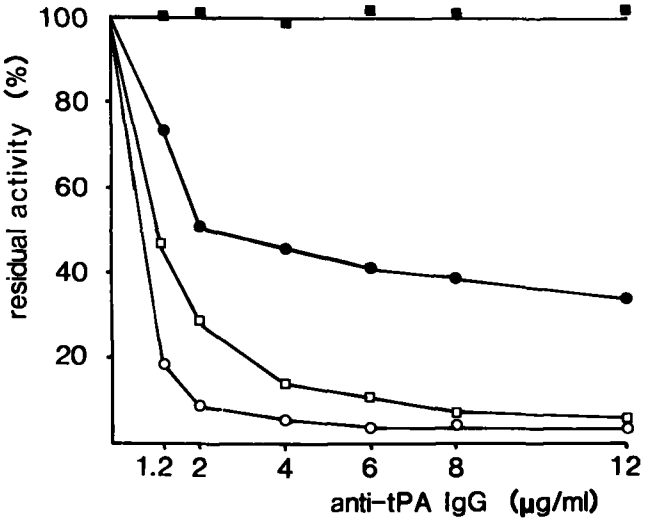
[1] Mean ± s.d.

[2] Significantly different from control nonperfused muscle ($p < 0.05$) by analysis of variance, followed by Bonferroni's modified t-test (29).

[3] Significantly different from control lung ($p < 0.05$) by Student's t-test.

The plasminogen activator in the tissue extracts was identified as t-PA by the following observations. PA activity in extracts was plasminogen- and fibrin-dependent. The IgG fraction of a rabbit anti-human t-PA-antiserum quenched human t-PA and the PA present in the tissue extracts (Fig. 3a). Some residual activity (corresponding to about 2-3 mIU/ml) always remained after quenching with anti-human t-PA. The amount of residual activity was, at maximal anti-t-PA IgG concentrations, independent of the amount of PA activity applied. When lung extract and muscle extract were assayed together, the residual activity was identical to the residual activity of the single extracts when assayed separately. This suggests that the residual activities observed were an artefact of the assay procedure, possibly due to an enhancement of the amidolytic activity of the trace amounts of plasmin present in the plasminogen preparation used. A similar effect has been described by Kluft et al. (21). Amiloride, a selective inhibitor of

(a)



(b)

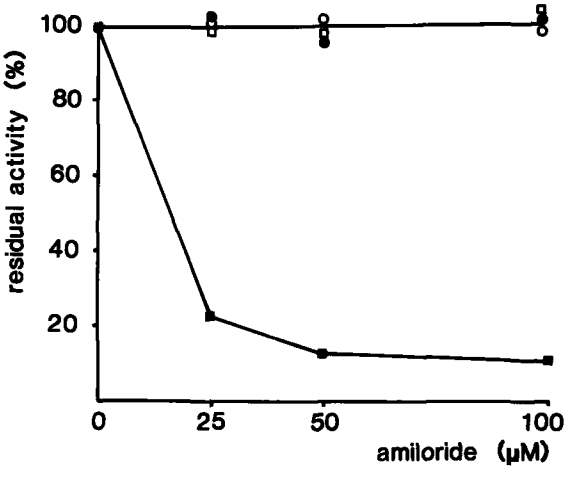


Fig. 3. Quenching of the plasminogen activator activity of human t-PA (○), rat muscle extract (●), rat lung extract (□), and rat urine (■) by anti-human t-PA IgG (Fig. 3a) and amiloride (Fig. 3b). Data shown are residual activities, as determined spectrophotometrically, after preincubation for 10 min with the indicated amount of IgG or amiloride. Amounts of activator applied were: human t-PA 50 mIU; rat muscle 10 mIU; rat lung 50 mIU; rat urine 2 µl.

urokinase-type PA (22), inhibited the PA activity of rat urine, but had no effect on the PA activity in lung or muscle extracts (Fig. 3b). The relatively high (35%; Fig. 3a) residual activity of muscle extracts in the presence of anti-t-PA IgG is, in view of the absence of an effect of amiloride (Fig. 3b) thus presumably due to the low level of activity in muscle extract, in combination with a constant residual activity.

After polyacrylamide gel electrophoresis, followed by fibrin autography, of lung and muscle extracts a major band of activity with a slightly slower mobility in the gel than the activity band of human t-PA was found (Fig. 4), in agreement with previous studies on the mobility of rat t-PA (8). In muscle extracts a second, much weaker band of activity was also found at a position corresponding to an M_r of approximately 100,000 (Fig. 4). The relative intensities of the two bands were not influenced by treatment with cycloheximide. No band of M_r = 100,000 was ever found in perfusate fractions (data not shown, compare refs. 8 and 9).

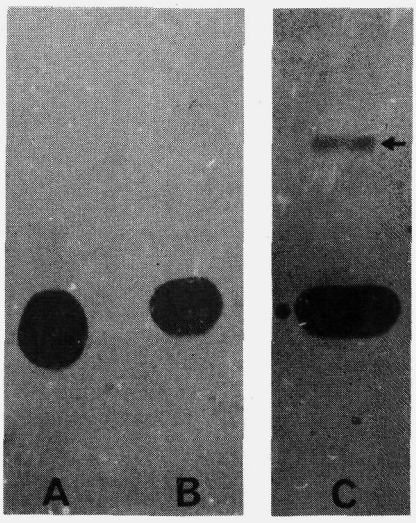


Fig. 4. Fibrin autography of human t-PA (lane A), rat lung extract (lane B) and rat muscle extract (lane C). Note that rat t-PA migrates slightly slower than human melanoma t-PA, in agreement with previous data (8). The weak band of activity at M_r = 100,000 in the muscle extracts (arrow) presumably represents t-PA-inhibitor complex.

DISCUSSION

A wide variety of procedures and chemical compounds can induce acute increases in the fibrinolytic activity of blood or, in perfused vascular beds, of the perfusing fluid (e.g. 4).

As has been discussed elsewhere (6), the increased activity is due to an acute increase in the t-PA concentration of blood or perfusion fluid. This increase, generally described as "t-PA release", is thought to be due to the active secretion (induced secretion, stimulation-dependent secretion; 23) of t-PA by endothelial cells. Other mechanisms are, however, theoretically possible (6). The acute increase of t-PA could be due to a rapid increase in t-PA synthesis (in combination with a constitutive secretion mechanism; 23) or due to an intracellular shunting of newly-synthesized t-PA from a degradative into a secretory pathway (24). A third possibility is that endothelial cells continuously secrete t-PA constitutively at a low level, resulting in a slow increase of the extracellular t-PA concentration in non-perfused parts of the vascular bed (e.g. some capillaries) or in the interstitium of the vessel wall. Stimulation by PAF or bradykinin could then result in the rapid wash-out of this extracellular pool from the tissues. Although this latter mechanism is unlikely in view of the regular perfusion of capillaries by plasma (25,26), no data refuting these three possibilities have been published.

The observations presented here show that inhibiting protein synthesis by cycloheximide did not affect t-PA release induced by PAF or bradykinin, either *in vivo* or *in vitro* in a perfused vascular bed, even if protein synthesis had been inhibited for 5 h. In perfused hindlegs, t-PA release was decreased in cycloheximide-pretreated animals only when PAF or bradykinin were applied as second stimulus, and not when these compounds were applied as first stimulus. Bradykinin, as a second stimulus, also induced less t-PA release in control animals. In all instances of reduced t-PA release, both oedema formation and a decrease in perfusate flow were noted. It is thus likely (in view of the correlation between t-PA release and perfusate flow, Fig. 1) that the reduction in t-PA release could (in these cases) be ascribed to an obstruction of part of the vascular bed, and not to a reduction in releasable t-PA. In a separate experiment (not shown) using untreated animals, PAF induced the release of comparable amounts of t-PA when perfusate flow was intentionally reduced from 10 ml/min to 7 or 4 ml/min. Flow rates above (the physiological) 10 ml/min were not studied. In view of the decreased flow, this means that the absolute amounts of t-PA released per min were decreased. The cause of this flow-depending effect is still unexplained. Pretreatment with cycloheximide for 3 h also did not affect the amount of t-PA released by PAF or bradykinin *in vivo* (Fig. 2). Of note is, however, that after 3 h of cycloheximide treatment dilute blood clots did not lyse within 24 h, while control clots lysed within 2 h (Fig. 2). It is thus possible that the source of releasable t-PA differs from that of t-PA circulating under base-line conditions, which might be derived from constitutively-secreted t-PA. This possibility is presently under investigation. These observations suggest that increased synthesis, intracellular shunting or the slow constitutive formation of an *extra*cellular pool are not involved in the observed acute increase in t-PA, leaving active secretion from a stable, pre-formed endothelial cellular storage pool as the most likely mechanism. The involvement of an

extracellular storage pool is also unlikely in view of the observations by Booyse et al. (27) and Kooistra and Emeis (28) that t-PA release can be induced *in vitro* in cultured endothelial cells. That t-PA is mainly present in or on endothelial cells (apart from some endocrine tissues) *in vivo* had already been demonstrated by histochemical (1,2) and immunohistochemical (3) techniques. The stability of this endothelial t-PA storage pool in muscle is shown in Table 3; no change was found in t-PA activity per mg of extracted protein. Although the extractable activity (expressed per g wet weight) decreased by 22% (not significantly), so did the amount of protein extracted (by 26%), suggesting that the decrease in extractable activity was due to oedema formation, not to a decrease in t-PA stores in muscle. In lung, however, the activity extracted per mg of protein decreased by 41% in 5 h. The stability of tissular stores of t-PA is thus not absolute. These conclusions are based on activity assays for t-PA, as no antigen assay for rat t-PA is available, and might thus be biased by changes in PA inhibitor concentrations. However, the intensity of the M_r = 100,000 activity band on fibrin overlays in muscle extracts (the tissue in which such a band was present) did not differ between control and cycloheximide-treated animals. This suggests that changes in PA inhibitor were of little importance, if present at all.

In previous experiments, calcium and arachidonic acid metabolism were shown to be involved in the acute t-PA release reaction (8). However, protein synthesis seems not to be required, as in endothelial cells stable stores of t-PA are present, which will be released after appropriate stimulation.

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CHAPTER 3

RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR IS INDUCED IN RATS BY LEUKOTRIENES C_4 AND D_4, BUT NOT BY PROSTAGLANDINS E_1, E_2 AND I_2

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1. Acute release of plasminogen activator (PA) was studied in rat isolated hindleg system perfused with Tyrode solution.
2. Leukotriene C_4(LTC_4) and LTD_4 dose-dependently induced the release of PA, which plateaued at 160 nmol l^{-1} and 200 nmol l^{-1}, respectively. The amount of PA released was about 1 iu ml^{-1}. The effects of LTC_4 and LTD_4 were not additive.
3. The PA released was identified as tissue-type PA (t-PA) by quenching experiments using anti-human t-PA IgG, by fibrin autography, and by the dependence of its activity on the presence of soluble fibrin.
4. LTE_4 (300 and 450 $nmol^{-1}$) and 5-hydroxy-eicosatetraenoic acid (600 nmol l^{-1}) did not induce any t-PA release in the perfusion system used.
5. Release of t-PA induced by LTC_4 and LTD_4 was inhibited by the leukotriene-receptor antagonist FLP 55712 (10 μmol l^{-1}), whereas FPL 55712 did not inhibit t-PA release induced by platelet-activating factor (Paf-acether).
6. *In vivo* LTC_4 and LTD_4 (2 μg kg^{-1} i.v.) also induced an acute increase of t-PA activity in rat blood as evidenced by decreased blood clot lysis times.
7. Prostaglandin E_1 and E_2, prostacyclin and the stable prostacyclin analogue ZK 36374 at concentrations of 0.1-3.0 μmol l^{-1} induced little or no t-PA release.

INTRODUCTION

The fibrinolytic and thrombolytic activity of blood is to a large extent determined by its content of plasminogen activators and their inhibitors. Of the various functionally and immunologically distinct types of plasminogen activator present in plasma (Emeis et al., 1985), only tissue-type plasminogen activator (t-PA) can show large and rapid changes

in plasma activity (Prowse & Cash, 1984; Emeis, 1987a). A better insight into factors regulating changes in blood t-PA levels might be of importance to develop means of manipulating blood fibrinolytic and thrombolytic activity pharmacologically.

In vivo, t-PA is synthesized and stored by vascular endothelial cells, which are considered to be the major, if not only, source of t-PA present in blood (Emeis, 1987a). From these endothelial cells, t-PA can be released into the blood, resulting in rapid and large increases in blood fibrinolytic activity (Prowse & Cash, 1984). The humoral and cellular mechanisms resulting in this acute release of t-PA are only vaguely understood (Emeis, 1987a). In a previous study we suggested, on the basis of inhibition studies, that products of a lipoxygenase pathway were involved in the acute release of t-PA from vessel walls (Emeis & Kluft, 1985).

In the present paper we will show that leukotriene C_4(LTC_4) and LTD_4, though not other eicosanoids, can induce in rats the acute release of t-PA, both *in vivo* and in a perfused hindleg vascular bed.

METHODS

Rat hindleg perfusion.

The rat perfused hindleg system was used to study the release of tissue-type plasminogen activator (t-PA) from a perfused vascular bed (Emeis, 1983). Male Wistar rats (Centraal Proefdierbedrijf TNO, Zeist, The Netherlands) weighing 220-300 g were anaesthetized with pentobarbitone (Nembutal, 60 mg kg^{-1} intraperitoneally). With the animal anaesthetized, the abdominal cavity was opened and the aorta and inferior vena cava were carefully dissected out. Ligatures were applied round the renal vessels and these were tightened to prevent leakage during the perfusion. Double ligatures were loosely applied round the aorta and vena cava separately. The upper ligature round the aorta was tightened and an 18-gauge needle was immediately inserted into the vessel and pushed distally up to the bifurcation. The lower ligature was then tightened securing the needle in place and the perfusion started by means of a constant flow roller pump. Next the upper ligature around the vena cava was tightened and the vessel was severed distal to the tied ligature to allow unimpeded outflow. The animal's thorax was opened and the pulmonary vessels severed to kill the animal. A cannula was inserted distally into the vena cava and tied into place. The rat hindlegs were perfused at a constant flow of 9 to 10 ml min^{-1} using Tyrode solution (composition in mmol l^{-1}. NaCl 146, KCl6, $CaCl_2$, 3, $MgCl_2$0.5, $KH_2PO_4$0.3, $NaHCO_3$20, glucose 5.6) containing 0.1 mg ml^{-1} bovine serum albumin (BSA), pH 7.4 at 37°C and oxygenated with 95% O_2 and 5% CO_2. Perfusion pressure was measured just proximal to the inflow by means of a mercury manometer. Each experiment was started with a 30 min perfusion of the Tyrode/BSA solution

through the hindleg region to clear the vessels from residual blood. Then compounds to be tested (see Results section) were added to the Tyrode/BSA and immediately perfused through the hindlegs. Sample collections were taken every 30 s for 30 s from the vena cava cannula and placed on ice. Subsequently, a 5 min wash with Tyrode/BSA solution was carried out before another compound, generally platelet-activating factor (Paf-acether), was perfused to test the responsiveness of the vascular bed. On completion of the experiment the samples were centrifuged (3000 g for 10 min), mixed at a 10:1 ratio with a solution containing 0.5 M Tris HCl (pH = 7.5) and 1% Triton X-100, and stored at -20 °C. This procedure stabilized the activity of the samples for at least four months (data not shown). The samples in 30 s blocks were usually analysed for t-PA activity immediately after experimentation.

To ensure that the hindleg region was totally perfused and no blockages had occurred, Evans Blue dye was injected into the inflow tube: the passage of the dye through all vessels indicated a complete perfusion.

Compounds and solvents.

Leukotrienes were obtained in a solvent composed of methanol, water, acetic acid and ammonium hydroxide (65:35:0.03:0.04) and added as such to the Tyrode/BSA. Prostaglandin E_1 (PGE_1) and PGE_2 were dissolved in ethanol, and PGI_2 in 0.1 N NaOH, and added to Tyrode/BSA immediately before perfusion. FPL 55712 and ZK 36374 were dissolved directly in the Tyrode/BSA. Paf-acether was prepared as described by Emeis & Kluft (1985).

Spectrophotometric plasminogen activator assay.

The PA activity of the sample was determined by the indirect spectrophotometric rate assay described by Verheijen et al. (1982). In brief: to wells of a 96-well microtiter plate were added: 75 μl buffer (0.1 mol l^{-1} Tris HCl, pH 7.65 containing 0.1% Tween 80), 20 μl soluble fibrin digest (1 mg ml^{-1}), 30 μl sample, 100 μl S-2251 (0.66 mmol l^{-1}) and 25 μl human plasminogen (1.11 μmol l^{-1}). The microtiter plate was incubated at 37 °C and after 45, 65, 85, 105 and 125 min; absorption was measured at 405 nm in a Titertek multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland). PA activity was then calculated according to Drapier et al. (1979). The detection limit for the assay was 0.09 iu ml^{-1}.

Dilutions of human melanoma t-PA (Kluft et al., 1983) were run in each plate for calibration. The PA activity of samples will be expressed in International Units (iu), as defined by the International Standard of t-PA (Gaffney & Curtis, 1985).

In some experiments either the soluble fibrin digest or the human plasminogen was omitted from the incubation mixture.

Quenching experiments.

The quenching experiments used the same spectrophotometric t-PA assay as mentioned above with the following addition: to the buffer, fibrin digest, and sample, 0-40 μl of a rabbit anti-human t-PA IgG solution was added. The plate was incubated for 10 min at 37 °C and then the substrate and plasminogen were added and the incubation proceeded as described above.

***In vivo* release of plasminogen activator.**

Rats were injected intravenously with LTC_4 or LTD_4 at a dose of 2 $\mu g\ kg^{-1}$ body weight. Leukotrienes (0.1 ml in solvent) were diluted with 0.9 ml of saline to a concentration of 1 $\mu g\ ml^{-1}$. Controls received only the solvent (2 ml kg^{-1}). Blood samples were obtained from a cannula in the carotid artery before and at 1, 2, 3, 5, 7 and 10 min after injection.

Blood (0.2 ml) was diluted to 10% in 1.7 ml of 0.12 mol l^{-1} sodium acetate (pH 7.4), clotted with 0.1 ml thrombin (20 u ml^{-1}) and incubated at 37 °C. Lysis times were read in minutes. When indicated, antibodies were added to the diluted blood 5 min before the addition of thrombin.

Fibrin autography.

Sodium dodecyl sulphate/8% polyacrylamide slab gels were prepared according to Laemmli (1970). Fibrin autography was performed according to Loskutoff & Mussoni (1983).

Materials.

All the chemicals used were of analytical grade. The products necessary for the spectrophotometric assay, such as the fibrin digest, substrate S-2251 and plasminogen were described previously (Verheijen et al., 1982). Conditioned medium from rat L_2 cells (Wewer et al., 1981) was used as a source of rat t-PA. Rabbit anti-human t-PA IgG (100 $\mu g\ ml^{-1}$) was prepared in our institute (Rijken et al., 1984).

The compounds used were obtained from the following sources: LTC_4, LTD_4 and LTE_4 as free acids from Paesel GmbH & Co, Frankfurt, West Germany; PGE_1, PGE_2, PGI_2 and bovine albumin (fraction 5) from Sigma, St. Louis, U.S.A.; ZK 36374 (5-(E)-(1S,5S,6R,7R)-7-Hydroxy-6-[(E)-(3S,4RS)-3-hydroxy-4-methyl-1-octane-6-inyl]bicyclo[3.3.0] octane-3-ylidenepentanoic acid) from Schering AG, Berlin, West Germany; FPL 55712 (sodium 7-[3-(4-acetyl-3-hydroxy-2-propyl-phenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate) from Fisons Pharmaceuticals, Loughborough, U.K.; bovine thrombin from Leo Pharmaceuticals, Ballerup, Denmark; Nembutal from Sanofi, Paris, France; Paf-acether from Bachem, Bubendorf, Switzerland; 5-hydroxy-eicosatetraenoic acid (5-HETE) from Unilever Research Laboratories, Vlaardingen, The Netherlands.

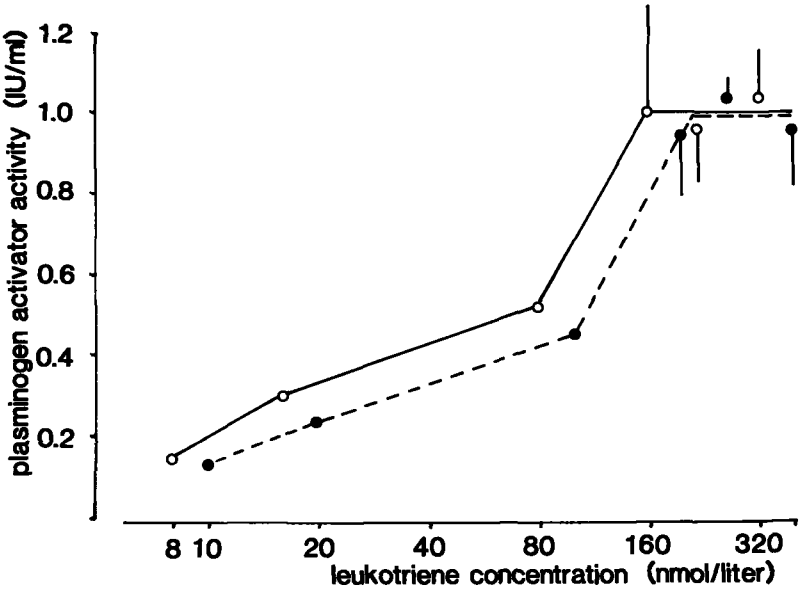

Fig. 1. Dose-dependent release of tissue-type plasminogen activator (t-PA) induced by leukotriene C_4 (LTC_4 ○) and LTD_4 (●) in perfused hindlegs of the rat. Data are shown as means with vertical lines indicating s.d. (n = 4), or as mean (n = 2) values, of t-PA concentrations in the 60-90 s sample blocks.

RESULTS

The release of PA by LTC_4 and LTD_4.

Perfusion using only Tyrode/BSA solution did not result in PA release. When LTC_4 (8-320 nmol l^{-1}) was added to the Tyrode/BSA solution and perfused through the rat hindleg region, analysis of the samples collected showed the presence of plasminogen activator (PA) in the perfusate. The amount of PA released was found to be dose-dependent, increasing and reaching a maximum at a dose of 160 nmol l^{-1} (Fig. 1). LTD_4 (10-400 nmol l^{-1}) induced PA release in a very similar fashion to LTC_4. The release of PA was also dose-dependent, in this case reaching a maximum at a dose of 200 nmol l^{-1}. The maximal amount of PA released was similar for LTC_4 and LTD_4, and amounted to about 1 iu ml^{-1} (Fig. 1). Both LTC_4 and LTD_4 followed a similar time course of PA release with peak values being always present in the 60-90 s sample block and decreasing gradually over the next few minutes (Fig. 2).

In two experiments, LTC_4 (160 nmol l^{-1}) and LTD_4 (200 nmol l^{-1}) were added together in the same perfusion buffer. The PA activity released by the combined leukotrienes (0.99, 0.90 iu ml^{-1}) was equivalent to the amount of PA activity released by each of them

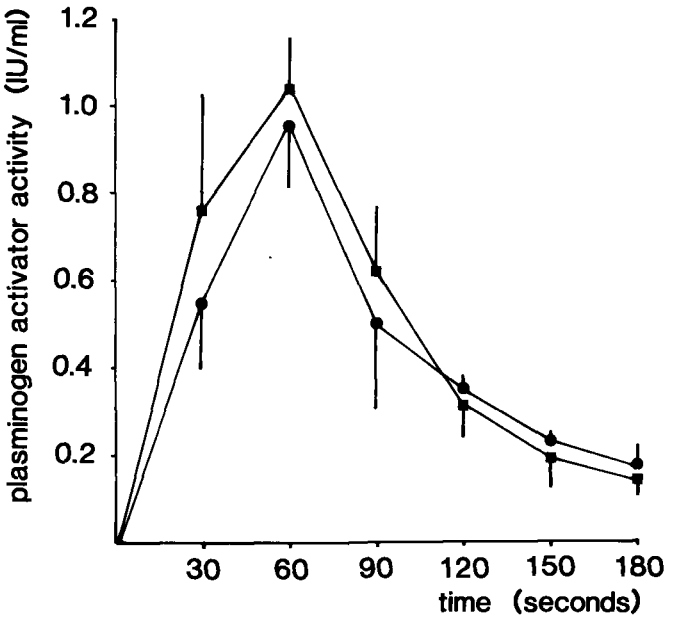

Fig. 2. Time course of tissue-type plasminogen activator (t-PA) release induced by leukotriene C_4 (■; 320 nmol l^{-1}) and leukotriene D_4 (●; 400 nmol l^{-1}) in perfused hindlegs of the rat. Each point represents the mean (n = 4) and vertical lines indicate s.d.

when given alone, and not equivalent to the sum of these amounts. In the perfusions using the leukotrienes no pressure changes or variations in the flow were noted. No oedema was seen in any of the animals following these perfusions. In contrast to LTC_4 and LTD_4, LTE_4 in concentrations of 300 and 450 nmol l^{-1} did not induce any PA release in the hindleg system (Table 1).

Perfusions were also done using 5-HETE, but no PA release was induced using this compound at a concentration of 600 nmol l^{-1} (Table 1). Paf-acether is known to induce the release of large amounts of t-PA in the hindleg model (Emeis & Kluft, 1985). To test each individual experiment and ensure that the animal was responsive, Paf-acether (20 nmol l^{-1}) was perfused for several minutes after each of the compounds investigated. In all the experiments, Paf-acether did induce t-PA release, whether the compound perfused previously to it had induced t-PA release or not. Following the perfusion with Paf-acether, some oedema was seen in the animal's hind quarter; a slight increase in pressure and decrease in the flow was also noted. In solvent control experiments no PA release was detected.

Table 1. Induction of tissue-type plasminogen activator (t-PA) release in the rat perfused hindleg system.

Compound	Concentration (nmol l^{-1})	n	t-PA release (iu ml^{-1})
LTC_4	320	4	1.04 ± 0.11*
LTD_4	400	4	0.94 ± 0.14
LTE_4	300	3	ND
	450	2	ND
5-HETE	600	5	ND
Solvent controls	-	5	ND
PGE_1	600	2	ND
	3000	2	< 0.10
PGE_2	3000	4	< 0.15
PGI_2	3000	3	< 0.10
ZK 36374	100	3	ND
	1000	3	ND
Paf-acether	20	4	2.79 ± 0.81

* Mean ± s.d. of maximal t-PA concentration obtained during perfusion of rat hindlegs in the presence of the indicated concentration of release-inducing compound. Maximal concentrations were always found in the 60-90 s sample block. ND = none detected.

Table 2. Effect of FPL 55712 on tissue-type plasminogen activator (t-PA) release in rat perfused hindlegs.

Compound	Concentration (nmol l^{-1})	n	t-PA release (iu ml^{-1})
LTC_4	160	4	1.00 ± 0.32*
LTC_4 and FPL 55712	160 10^4	4	ND
LTD_4	200	3	0.94 ± 0.11
LTD_4 and FPL 55712	200 10^4	3	ND
PAF	20	4	2.79 ± 0.81
PAF and FPL 55712	20 10^4	6	3.01 ± 0.79

* Mean ± s.d. of maximal t-PA concentration obtained (in the 60-90 s blocks) by perfusing rat hindlegs with the indicated concentration(s) of compound(s). ND = none detected.

The effect of FPL 55712 on PA release.

To investigate the mode of release of PA by LTC_4 and LTD_4, FPL 55712, a leukotriene receptor antagonist (Augstein et al., 1973; Musser et al., 1986), was used (Table 2). FPL 55712 (10 μmol l^{-1}) was added to the Tyrode/BSA buffer and perfused through the hindleg region for 20 min before the addition of either LTC_4 (160 nmol l^{-1}) or LTD_4 (200 nmol l^{-1}). FPL 55712 totally blocked the release of PA by both LTC_4 and LTD_4. However, it did not affect the release of PA induced by Paf-acether and so was not an inhibitor of the release reaction.

Determining the type of PA released.

The activator released by LTC_4 and LTD_4 was identified as tissue-type PA (t-PA) by the following observations.

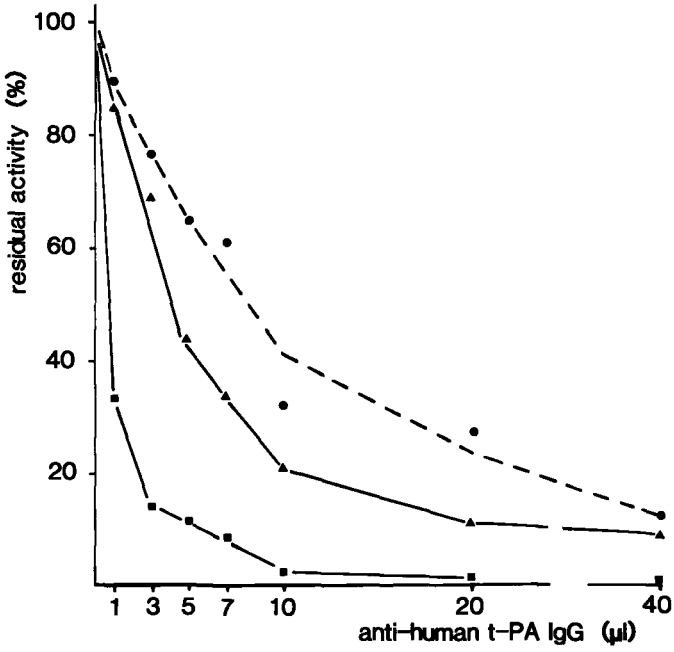

Fig. 3. Quenching of plasminogen activator (PA) activity in perfusate samples by anti-human PA tissue-type (t-PA) antibodies. Perfusate samples (30 min from leukotriene C_4 (LTC_4)- or LTD_4-stimulated rat hindlegs were incubated with increasing amounts of rabbit anti-human t-PA IgG (100 μg ml^{-1}) for 10 min at 37°C, and subsequently the residual PA activity was determined spectrophotometrically. Data shown are means of duplicate determinations: the percentages of residual activity for LTC_4- and LTD_4-induced perfusates are averaged (▲), as these percentages did not differ by more than 4%. Also shown for comparison is the quenching by anti-human t-PA Ig of human t-PA (■; 30 min) and of rat L_2 t-PA (●); 60 min).

Firstly, a series of quenching experiments were done using the IgG fraction of a rabbit anti-human t-PA antiserum. The specific anti-human t-PA IgGs quenched human t-PA, rat t-PA and the PA present in the perfusate samples dose-dependently (see Fig. 3). As expected, heterologous rat t-PA was quenched less efficiently than the homologous human t-PA.Secondly, the activation of plasminogen by t-PA is enhanced by soluble fibrin(ogen) fragments whereas plasminogen activation by urokinase-type plasminogen activators is not (Verheijen et al., 1982). Several spectrophotometric assays were done

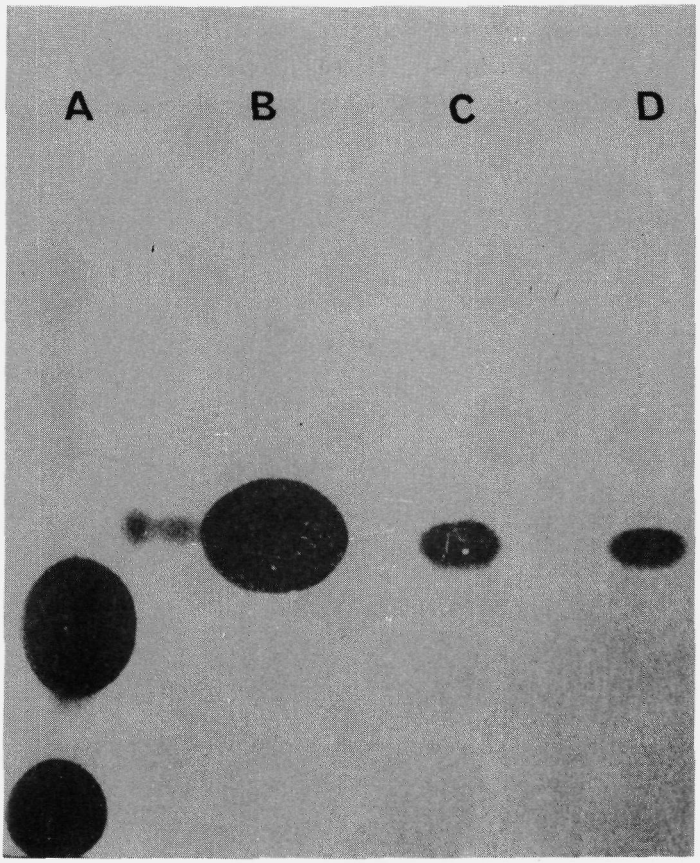

Fig. 4. Fibrin autography of perfusate samples from leukotriene-stimulated rat hindlegs. Human urokinase (lane A), rat L_2 tissue-type plasminogen activator (t-PA, lane B) and perfusate samples after stimulation with 160 nmol l^{-1} of leukotriene C_4 (LTC_4, lane C) or 200 $nmol^{-1}$ of LTD_4 (lane D) were electrophoresed in 8% polyacrylamide slab gels (Laemmli system). The gel was then soaked for 2 h in 2.5% Triton X-100 (with one change), washed, and placed on top of a plasminogen-rich fibrin-agarose gel. The dark areas indicate lysis of the fibrin gel, caused by PA diffused from the polyacrylamide gel into the fibrin gel. The mobility of the PA-activity in the perfusate samples is identical to that of rat t-PA (M_r = 70,000), but different from that of urokinase (M_r = 55,000 and 33,000).

omitting the soluble fibrin digest; plasminogen activation by the perfusate samples was only detectable in the presence of soluble fibrin fragments.
Thirdly, in fibrin autography (Fig. 4) both rat t-PA from L_2 cells and perfusate samples of LTC_4 and LTD_4 showed a single lysis zone at the molecular weight of approx. 70,000, identical to the M_r of human t-PA, but different from that of urokinase-type PA (molecular weights 55,000 and 33,000).

***In vivo* experiments.**

To see whether LTC_4 and LTD_4 would also induce t-PA release *in vivo*, each compound was injected intravenously into rats. Both LTC_4 and LTD_4 (2 $\mu g\ kg^{-1}$) were found to increase blood fibrinolytic activity, as evidenced by decreased blood clot lysis times (Fig. 5). At one min after injection, dilute blood clot lysis times were decreased by 86 ± 14

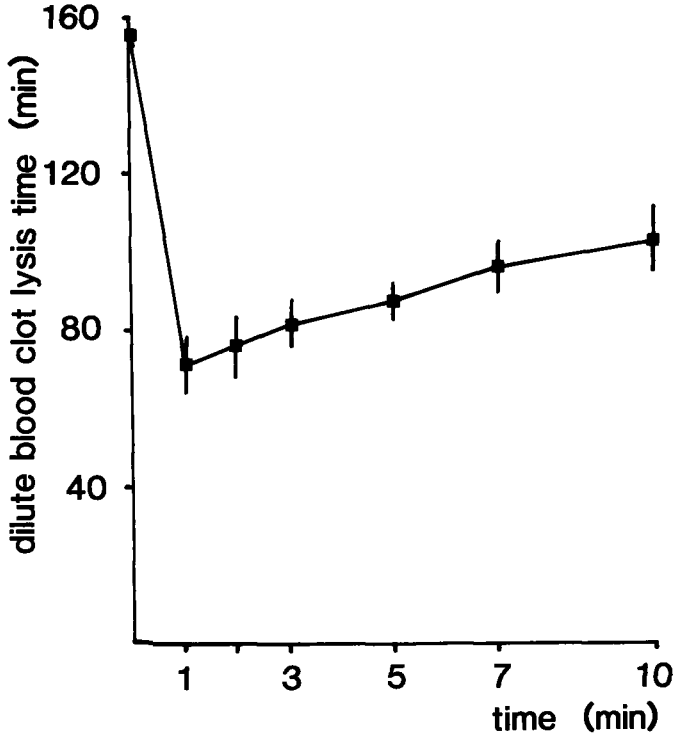

Fig. 5. (a) Time course of dilute blood clot lysis times after injection of leukotrienes. Data are shown as mean lysis times from four rats (two injected with leukotriene C_4 (LTC_4), 2 $\mu g\ kg^{-1}$; two with LTD_4, 2 $\mu g\ kg^{-1}$); vertical lines indicate s.d.

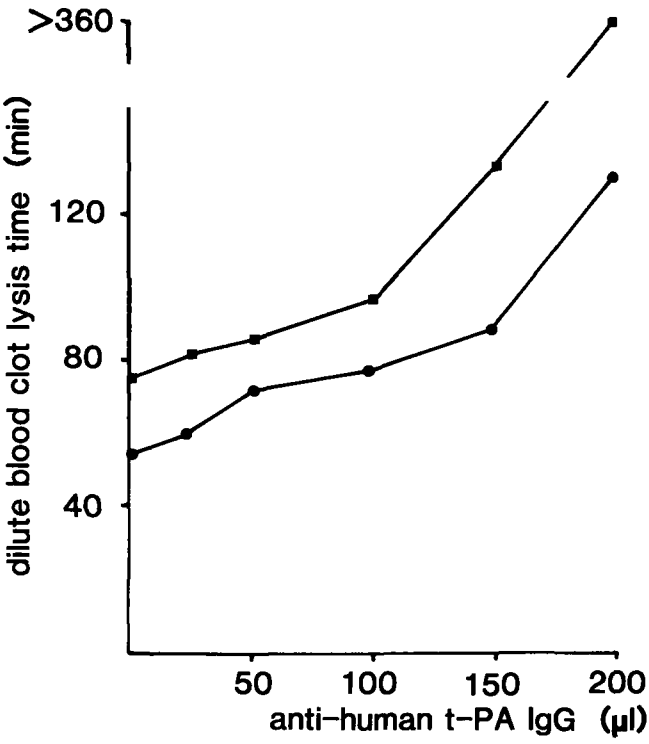

Fig. 5. (b) Incubation of blood from a LTC_4- and a LTD_4-injected rat with anti-human tissue-type plasminogen activator (t-PA) IgG normalizes the decreased dilute blood clot lysis times. Blood was obtained from rats one minute after the i.v. injection of leukotriene C_4 (■) or D_4 (●), both at a dose of 2 $\mu g\ kg^{-1}$.

min in the leukotriene-treated animals, as compared to 7 ± 13 min (n = 5) in the control, solvent-treated, rats. No decreased blood clot lysis times were seen with the solvent controls. The increase in blood fibrinolytic activity could be quenched by pre-incubation of the diluted blood with antibodies against human t-PA (Fig. 5).

Investigation of other eicosanoids.

Several cyclo-oxygenase products were also tested in the hindleg perfusion system (see Table 1). PGE_1 was perfused at concentrations of 600 nmol l^{-1} and 3 μmol l^{-1}. Whereas no PA was detected at the lower dose, at the higher dose a very small amount of t-PA release was induced. PGE_2 (3 μmol l^{-1}) and PGI_2 (3 μmol l^{-1}) also induced the release of small amounts of t-PA, which were significantly less than those released by LTC_4 and

LTD_4.
ZK 36374, a stable prostacyclin analogue (Schrör et al., 1981), was also perfused through the hindleg system (at 100 nmol l^{-1} and 1 μmol l^{-1}) but no PA release was detected.

DISCUSSION

Peptidoleukotrienes affect the vascular system in various ways. Injection of leukotrienes C_4 or D_4 into rats results in a short-lived increase in blood pressure, followed by hypotension (Piper, 1983; Feuerstein, 1984). In cultured vascular endothelial cells LTC_4 shows high-affinity plasma membrane binding, which can be inhibited by FPL 55712 (Chau et al., 1986). Moreover, LTC_4 and LTD_4, but not LTB_4 or LTE_4, stimulate endothelial cell prostacyclin synthesis (Benjamin et al., 1983; Cramer et al., 1983; Pologe et al., 1984; Clark et al., 1986a), presumably because leukotrienes activate phospholipase A_2 in endothelial cells (Clark et al., 1986b,c). Not only prostacyclin synthesis, but synthesis of Paf-acether as well, is enhanced by LTC_4 and LTD_4 though again not by LTE_4 or LTB_4 (McIntyre et al., 1986). Endothelial cells *in vitro* can also degrade LTC_4 into LTD_4 and LTE_4 (Pologe et al., 1984). Whether endothelial cells can synthesize leukotrienes from arachidonic acid is, however, doubtful (Feinmark & Cannon, 1986). Together these observations show that endothelial cells respond to LTC_4 and LTD_4, both *in vivo* and *in vitro,* and can convert LTC_4 into LTD_4 and LTE_4. In our constant flow-perfused hindleg system of the rat, LTC_4, LTD_4 and LTE_4 did not change perfusion pressure and did not cause oedema formation within the experimental time period, in agreement with the relatively small effects of hindleg blood flow and vascular resistance described by Eimerl et al. (1986) after *in vivo* injection of graded doses of leukotrienes.

LTC_4 and LTD_4 were approximately equi-effective in inducing the release of t-PA, while LTE_4 had no effect at all. The combined application of both LTC_4 and LTD_4 did not result in an enhanced release of t-PA compared to the release induced by LTC_4 or LTD_4 separately. In combination with the complete suppression of t-PA release by FPL 55712, the data suggest that both LTC_4 and LTD_4 interact with a single receptor on endothelial cells, binding to this receptor being sensitive to inhibition by FPL 55712. In cultured bovine endothelial cells, an LTC_4 receptor with K_D = 6.8 nM has been described (Chau et al., 1986). The dose-response curve obtained in our system is compatible with the presence of a similar receptor for LTC_4 and LTD_4 on rat vascular endothelial cells *in vivo.* Our data, however, cannot exclude the (quantitative) conversion of LTC_4 into LTD_4 during passage through the hindleg vascular bed, the t-PA release response being then due to activation of an LTD_4-receptor. Rats are indeed able to metabolize LTC_4 into LTD_4 efficiently (Denzlinger et al., 1985). To decide if a similar conversion is of

importance in our experimental system, we will have to await the availability of compounds interfering with the said conversion, or of receptor antagonists specific for LTD_4, rather than LTC_4 (Cheng, 1986; Lee et al., 1984; Musser et al., 1986).

The PA released by leukotrienes proved to be tissue-type PA, as demonstrated by the dependence of its activity on soluble fibrin, by the quenching of its activity by anti-human t-PA antibodies, and by its molecular weight, which was similar to that of cell culture-derived rat and human t-PA, but different from urokinase. Paf-acether and a variety of other compounds also induce the release exclusively of t-PA from perfused rat hindlegs (Emeis, 1983; Emeis & Kluft, 1985) and pig ears (Klöcking et al., 1984). Release of another type of PA, e.g. urokinase, has so far not been observed. As endothelial cells are the only cells in hindlegs to contain t-PA, the induced release of t-PA must be due to release of t-PA from vascular endothelial cells. In a previous paper (Emeis & Kluft, 1985) we showed that lipoxygenase inhibitors (and diethylcarbamazine, a putative peptidoleukotriene synthesis inhibitor, Bach & Brashler, 1986) inhibited Paf-induced t-PA release. As shown by others, in perfused rat lung (Voelkel et al., 1982) and heart (Piper & Stewart, 1986) Paf-acether does indeed cause increased leukotriene concentrations in the perfusate, the cellular origin of which is not known. The maximal amount of t-PA that could be released by LTC_4 or LTD_4 (about 1 iu ml^{-1}) was, however, less than the amount released by 20 nM Paf-acether (about 3 iu ml^{-1}). Also, in contrast to leukotriene-induced release, Paf-acether-induced t-PA release was not inhibited by FPL 55712. Together these two observations suggest that Paf-acether does not induce t-PA release exclusively by inducing the vascular synthesis and release of leukotrienes, which then cause endothelial cells to release t-PA.

The present study suggests that the effects on t-PA release of the lipoxygenase inhibitors and of diethylcarbamazine cannot be explained by decreased peptidoleukotriene release from the vessel wall. Whether other products of a lipoxygenase pathway (e.g. hydroxy-eicosatetraenoic acids or hydroxy-linoleic acid) or a mono-oxygenase pathway are involved in t-PA release, remains to be determined, although the data make a major role of 5-HETE unlikely. Neither can we exclude that leukotrienes synthesized intracellularly behave differently from exogenously supplied leukotrienes. However, a recent study on the effects of lipoxygenase inhibitors cautions against aspecific inhibitory effects of these compounds on secretion (Razin et al., 1984).
The observation that intravenous injection of LTC_4 or LTD_4 also induced acute release of t-PA, as evidenced by decreased dilute blood clot lysis times, shows that the induction of t-PA release *ex vivo* in a perfused vascular bed is not an artifact induced by the experimental procedure, but that leukotriene-induced t-PA release may be of physiological significance.

In perfused hindlegs, prostaglandins E_1 and E_2 and prostacyclin were unable to induce t-PA release, in agreement with previous negative results on the induction of PA-release by prostacyclin (Nakajima, 1983) and prostaglandins (Markwardt & Klöcking, 1978) in perfused ears of the pig. However, Hussaini & Moore (1985) demonstrated that in rats *in vivo* prostacyclin (and its metabolite 6-keto-PGE_1) induced increased fibrinolytic activity. Maximally increased activity was found at 30-60 min after injection, suggesting that the observed induction of increased fibrinolytic activity by cyclo-oxygenase products may proceed by a pathway different from acute t-PA release (as induced by e.g. leukotrienes) which generally peaks at one min after injection.
Other mechanisms that might be involved in prostacyclin-induced increased fibrinolytic activity (for discussion, see Emeis, 1987b) are now under investigation. Whether the peptidoleukotriene-induced release of t-PA from endothelial cells is of (patho) physiological significance in processes involving increased leukotriene production also remains to be determined.

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CHAPTER 4

THE SIMULTANEOUS ACUTE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND VON WILLEBRAND FACTOR IN THE PERFUSED RAT HINDLEG REGION

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SUMMARY

In perfused rat hindlegs, platelet-activating factor and bradykinin induced the acute release of both tissue-type plasminogen activator (t-PA) and von Willebrand Factor (vWF). The time course of release was similar for both proteins, and the amounts of t-PA and vWF released under various conditions were closely correlated. Release of both t-PA and vWF required extracellular calcium, and could be induced by the calcium ionophore A-23187. Protein synthesis was not required for release to occur.
Phorbol myristate acetate also induced release of t-PA and vWF, though with a different time course; DDAVP was inactive.
The results suggest that the release of t-PA, and that of vWF, are closely linked at the cellular level.

INTRODUCTION

Von Willebrand Factor (vWF) is a plasma glycoprotein that is biosynthesized and secreted by endothelial cells, and is necessary for platelet adhesion to the subendothelium when vascular injury occurs. Endothelial cells are known to store vWF in the Weibel-Palade body, an organelle specific for these cells (1,2,3).
vWF can be acutely released from its endothelial storage pool into the circulation (4,5). In cultured human umbilical vein endothelial cells vWF will be acutely released upon stimulation by thrombin (6-8), calcium-ionophore A-23187 (2,7-11), or phorbol ester (2,3,7-9). Acute release of vWF has also been observed upon stimulation of cultured

endothelial cells with plasmin, interleukin-1, adrenaline and bradykinin (12,13), histamine (11), endotoxin (14) and fibrin (15).
Another glycoprotein found in endothelial cells that also plays an important role in haemostasis is tissue-type plasminogen activator (t-PA), necessary for the activation of plasminogen to plasmin for clot lysis. Like vWF, t-PA is also released from endothelial cells upon stimulation by a wide variety of different compounds (16,17).
In previous studies we have shown the acute release of t-PA in the perfused rat hindleg by compounds such as histamine, thrombin, adrenaline and bradykinin (18), platelet-activating factor (PAF) (19) and leukotriene C4 and D4 (20).
In humans both t-PA and vWF are simultaneously released into the blood by various stimuli, such as venous occlusion, exercise and infusion of adrenaline and 1-desamino-8-D-arginine vasopressine (DDAVP) (21-25).
In this paper we have investigated whether in our experimental model of the perfused rat hindleg the induction of acute release of t-PA was also accompanied by release of vWF. As this proved to be the case, we used this system to explore mechanisms involved in the release of vWF and t-PA under *ex vivo* conditions.

MATERIALS AND METHODS

Methods.

Male Wistar rats (Broekman Institute, Helmond, The Netherlands), weighing 250-350 g, anaesthetized using pentobarbital (Nembutal®, 60 mg/kg intraperitoneally), were used in the following experiments.

Experimental model.

The perfused rat hindleg system was used to study the acute release of t-PA and vWF from a perfused vascular bed, as explained in detail elsewhere (20). Briefly: the rat hindleg was perfused through the aorta with oxygenated Tyrode's salt solution containing 0.1 mg/ml bovine serum albumin (BSA), pH 7.5, at 37°C using a roller-pump at a constant flow of 9-10 ml/min. Perfusate samples were collected from an out-flow cannula inserted into the vena cava. A 30 min perfusion period using Tyrode/BSA solution was allowed to clear residual blood from the blood vessel system. Routinely, two compounds were used to stimulate release; each was perfused through the system for 5 min with a 5 min wash-out period in between. Sample collections were taken every minute for 30 sec, kept on ice until the experiment was completed, and then centrifuged (3,000 × g for 10 min). The supernatant was collected and mixed 1:9 with a solution containing 0.5 M Tris/HCl (pH 7.5) and 1% Triton X-100, and either assayed immediately or stored at -20°C.

Spectrophotometric plasminogen activator assay.

The PA activity of the samples was determined by the indirect spectrophotometric rate assay described by Verheijen et al. (26). Sample volumes of 30 μl for the perfusate samples were used. Dilutions of human melanoma t-PA were run in each plate for calibration, and the activity of the samples will be expressed in International Units (IU), as defined by the International Standard for t-PA.

ELISA determination for von Willebrand factor.

The presence of vWF in the samples was determined using an ELISA assay adapted from Ingerslev (27). Flat-bottomed 96-well microtiter plates (Flow Laboratories, Irvine, UK) were coated with 100 μl of a 1:10,000 dilution in carbonate buffer (pH 9.6) of rabbit anti-human vWF immunogolublin (Dakopatts, Denmark) (28) and left overnight at 4 °C. PBS-Tween buffer (pH 7.2, containing 0.05% Tween 20 and 0.05% BSA) was used to wash the plates before the perfusate samples (25 μl and 75 μl of PBS-Tween buffer) were added. After 2 h, bound antigen was detected using peroxidase conjugated rabbit immunoglobulins to human vWF (Dakopatts), diluted 1:3,000 in PBS-Tween buffer. After incubation for a further 2 h at room temperature, the plates were treated with 100 μl of the chromogenic substrate and the reaction was stopped after 20 min with 25 μl 4 N H_2SO_4. Absorbances were measured at 450 nm in a Titertek multiscan spectrophotometer (Flow Laboratories, Irvine, UK). Rat pooled plasma in a range of 0.05 to 5% was used in each plate for calibration. The concentration of vWF will be expressed as units, hundred units being equivalent to the amount of vWF present in 1 ml of pooled rat plasma. The detection limit of the assay was 0.1 U/ml.

***In vivo* release of von Willebrand factor.**

Rats were injected intravenously with PAF (1 μg/kg), endotoxin (1 mg/kg) or saline (2 ml/kg). Blood samples were obtained from a cannula in the carotid artery before and at 3, 5, 10, 20 and 30 min after injection. The blood was anticoagulated with citrate and platelet-poor plasma was prepared at 4 °C and stored at -20 °C. The plasma (diluted 1:100) was assayed by ELISA to determine the concentration of vWF.

Materials.

Platelet-activating factor (PAF) was obtained from Bachem (Bubendorf, Switzerland), Nembutal® was from Sanofi (Paris, France); bradykinin, bovine serum albumin, cycloheximide, calcium ionophore A-23187 (free acid), phorbol 12-myristate 13-acetate and endotoxin (E. coli 0128:B12) were from Sigma (St. Louis, MO). DDAVP (1-desamino-8-D-arginine-vasopressine) was a gift from Ferring AB (Malmö, Sweden). Purified human t-PA (two-chain) was a gift from Dr. J.H. Verheijen of the Gaubius

Institute. The International Standard for t-PA (83/517) was obtained from the National Institute for Biological Standards and Control (London, UK).
The materials used in the spectrophotometric PA assay have been detailed elsewhere (26). All other materials were of analytical grade.

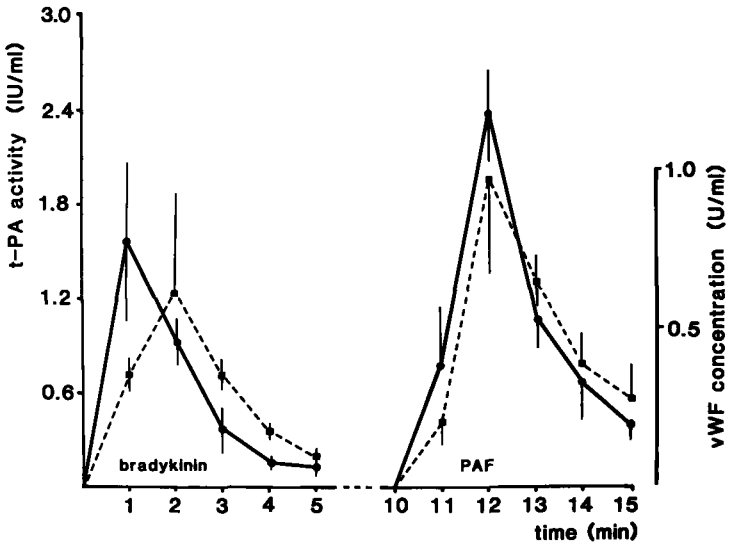

Fig. 1. Time course of t-PA activity (●—●) and vWF concentration (■---■) in perfusates from rat hindlegs during stimulation with bradykinin (0.8 μM) for 5 min, followed - after a 5-min wash - by a 5-min stimulation with PAF (5 nM). Data shown are mean ± s.d. of four perfusions.

RESULTS

Comparison of the release of t-PA and von Willebrand factor.

t-PA and vWF were assayed in the same perfusion samples and were found to follow a very similar pattern of release (see Fig. 1). Neither t-PA nor vWF showed any detectable baseline releaee in the system before stimulation. On stimulating with bradykinin (0.8 μM), the first release-inducing compound in these experiments, t-PA levels reached a peak in the first minute, while vWF levels peaked in the second minute of the 5-min perfusion period. The concentration of both vWF and t-PA then declined during the subsequent minutes of stimulation. At the end of the 5 min wash-out period, t-PA and vWF were again undetectable. When using PAF (5 nM), the second release-inducing compound we studied, both t-PA and vWF release peaked in the second-minute sample collections (Fig. 1). The amounts of t-PA and vWF released by a 5 nM PAF stimulation were always found to be larger than the amounts released by a 0.8 μM bradykinin

stimulation. When the sequence of inducers was reversed, identical observations on t-PA and vWF release were made (not shown; compare 32).

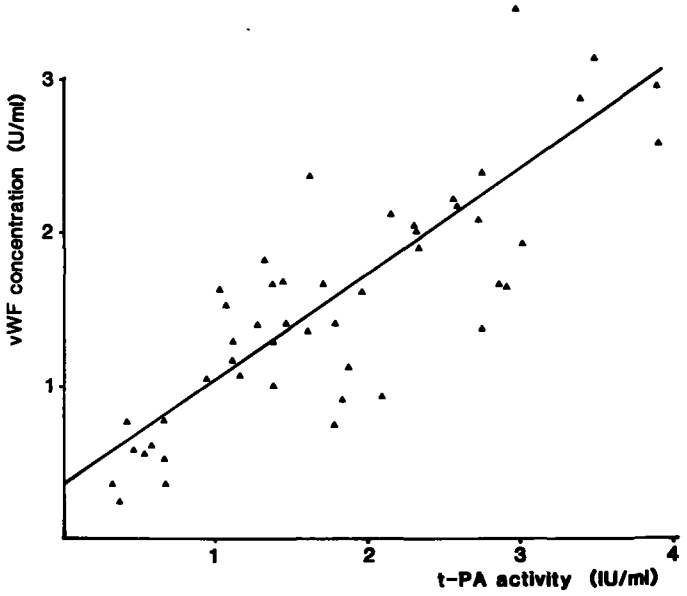

Fig. 2. Diagram depicting the relationship between peak levels of t-PA activity and of vWF concentration during induced release. Data points from 48 perfusions are shown; r = + 0.824 ($p < 0.001$).

Concentration of both t-PA and vWF released by either bradykinin or PAF are close within a group of rats in a set experiment, but may vary from one group of rats to the next. A collection of 48 peak samples from different perfusion experiments, including various concentrations of PAF or bradykinin, were assayed for both t-PA and vWF. Fig. 2 shows a diagram comparing maximal t-PA release to vWF release in these 48 individual perfusion experiments. The correlation coefficient was 0.824 ($p < 0.001$), showing that the release of t-PA and vWF are not only correlated in time (e.g. Fig. 1), but also correlated quantitatively. DDAVP (0.1 μg/ml) did not induce the release of vWF (data not shown). As we reported before (18), DDAVP did not induce the release of t-PA either.

***In vivo* experiments.**

From previous work, PAF is known to induce the release of t-PA *in vivo* as well (19,20) and this increased release is seen within the first minute after i.v. injection. Using an i.v. injection of PAF (1.0 μg/kg), the effect of PAF on vWF release *in vivo* was studied (Fig. 3).

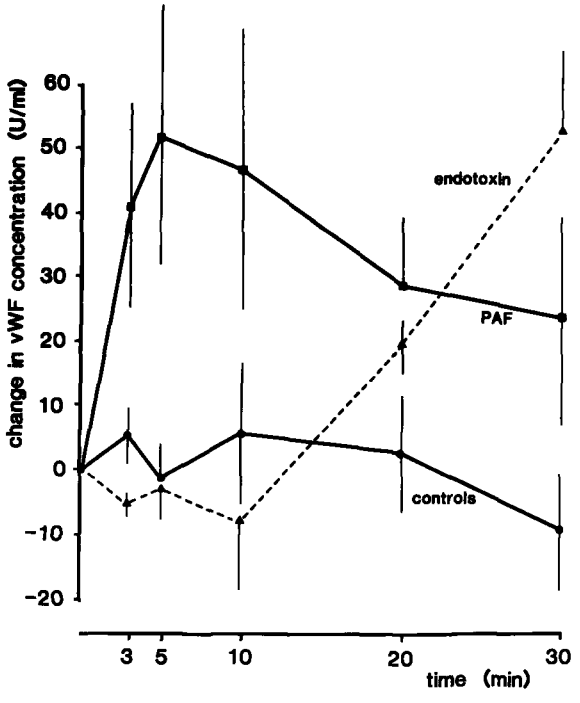

Fig. 3. Time course of changes in plasma concentration of vWF in rats after i.v. injection of either saline (n = 6) (●—●), PAF (1 μg/kg; n = 3) (■—■) or endotoxin (1 mg/kg; n = 3) (▲---▲). Data shown are mean ± s.e.m. of changes in vWF plasma concentration, relative to a control plasma sample obtained before injection of compound.

The level of vWF in the plasma samples increased with time in the PAF-injected animals, but not in the saline controls. The peak levels were reached in the samples collected at 5-10 min after injection, and decreased slowly thereafter. Endotoxin induced, after a lag period of 10 min, a gradual increase in vWF concentration (Fig. 3; compare refs. 29-31). DDAVP (5 μg/kg) had no effect (data not shown).

Effect of Ca^{2+} on the stimulus-induced release of t-PA and vWF.

The role of extracellular calcium in the release of vWF was investigated, because t-PA release (19), and the release of vWF from cultured endothelial cells (8) both have been shown to require extracellular calcium. We performed experiments using the rat perfused hindleg model with two different stimulations, both in the presence and absence of Ca^{2+}; during the experiments without calcium the Tyrode buffer also contained 1 mM disodium EDTA. This buffer was perfused through the animal for 20 min before stimulation to ensure the complete absence of extracellular calcium. Using the bradykinin stimulation, both t-PA and vWF release in the absence of Ca^{2+} were reduced to 25% of the control values. During PAF stimulation, a 95% inhibition of both t-PA and vWF release was seen, the release of both glycoproteins being affected to the same degree (data not shown).

Effect of the calcium ionophore A-23187 and of a protein kinase C activator on t-PA and vWF release in perfused rat hindlegs.

In the next set of experiments, the calcium ionophore A-23187 was tested for induction of t-PA and vWF release.

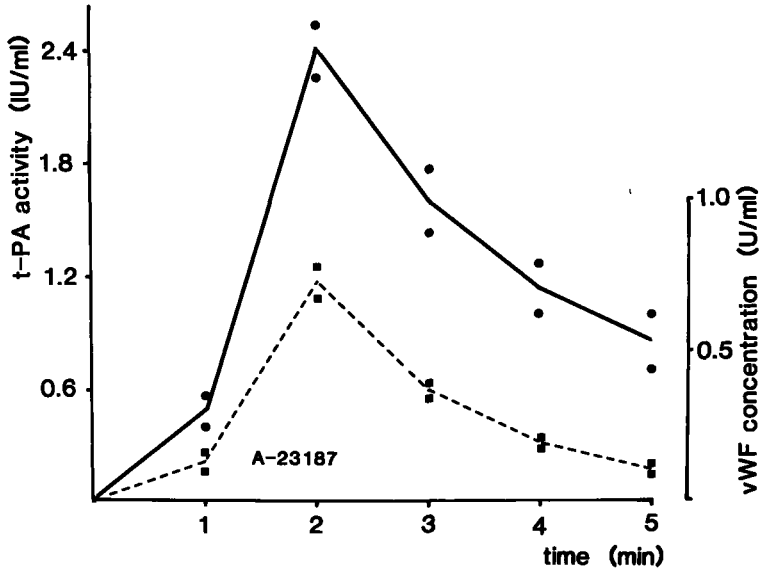

Fig. 4. Time course of the release of t-PA activity (●—●) and of vWF (■---■) from perfused hindlegs during stimulation with the calcium ionophore A-23187 (2 μM). Data points from two experiments are shown.

As shown in Fig. 4, A-23187 (2 μM) did induce both t-PA and vWF release. The time-course of A-23187 induced release of both glycoproteins was similar to that induced by other release-inducing compounds.

Investigating the possible role of protein kinase-C (PK-C) in acute vWF release, the PK-C activator, phorbol 12-myristate 13-acetate (PMA, 100 nM), was added to the perfusion fluid.

PMA induced t-PA and vWF release which did not peak, but remained stable for the 15 min of perfusion (Fig. 5). The concentration of t-PA released by PMA was about 0.3 IU/ml, while the concentration of vWF released averaged 0.3 U/ml. The ethanol solvent controls did not induce any vWF or t-PA release.

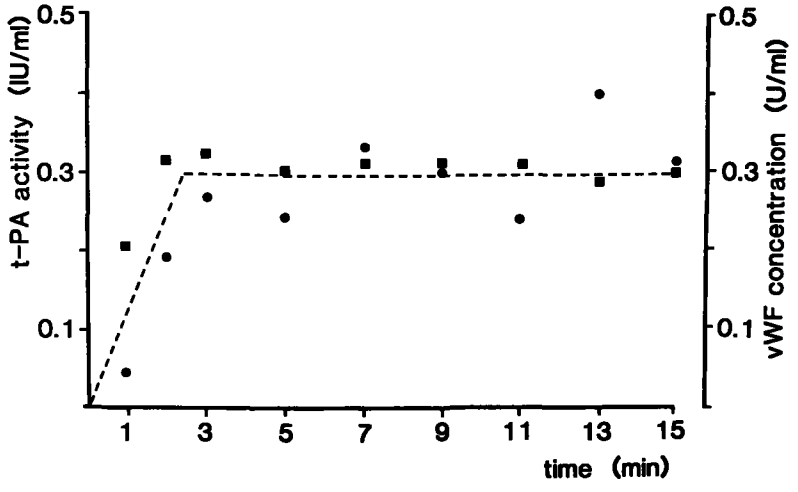

Fig. 5. Concentrations of t-PA (■) and vWF (●) released from rat hindlegs during a 15-min perfusion with PMA (100 nM). Data shown are mean values (n = 4). Solvent controls (0.1% v/v ethanol) showed no detectable release of either protein.

DISCUSSION

The experiments described in this paper show two main findings: firstly, the time course of acute release of vWF is similar to that of t-PA in a perfused rat system (18-20) for a variety of compounds and, secondly, that the release of vWF is quantitatively correlated with t-PA release for a given compound. In our model there is no measurable baseline

release of either glycoprotein prior to the addition of a release-inducing stimulus. Once the stimulus has been added to the system, the reaction is very fast: the peak release point is seen after 1 or 2 min and release then gradually decreases to baseline levels over a 5 min period (e.g. Figs. 1 and 4). An explanation for the decrease of t-PA activity after the peak of induced release might be a - slower - induced release of PA inhibitor. We have, however, never detected any PA inhibitor activity in perfusate samples, either before, during or after the induction of t-PA release (unpublished observation). Nor have we, by fibrin autography, ever detected t-PA-PA inhibitor complexes in perfusate samples (as illustrated in refs. 19 and 20). A final solution to this problem would require measurement of rat t-PA antigen; our present rat antigen assay is, however, still not sensitive enough to be useful in this respect.
After a 5 min wash-out period, there is no t-PA or vWF detectable in the perfusate samples, but another stimulus-induced compound will again release both glycoproteins. Release in this system is thus a rapid reaction which can be termed an acute release reaction.

Previous studies, using the protein synthesis inhibitor cycloheximide, indicated that the t-PA released in such an acute reaction comes from a stable endothelial cell storage pool (32). Similar experiments were done, using cycloheximide, to test for the release of vWF from the perfused rat hindleg. The results (not shown) demonstrated that identical amounts of vWF were released after pretreatment of rats with cycloheximide for 3 h, so indicating that the stimulus-inducible secretion originates from a stable vWF storage pool in the endothelial cells and not from recently synthesized vWF.

The presence of a storage pool of vWF, the Weibel-Palade body, and the release of vWF from these particles by stimulation of cultured endothelial cells, has been shown by several groups (e.g. 1-3).
The acute release of both t-PA and vWF was found to be dependent on the presence of Ca^{2+} in the perfusion buffer. Using a Ca^{2+}-free Tyrode buffer, the release response of both glycoproteins was strongly decreased compared to that in the controls. Using cultured endothelial cells, Loesberg et al. (7) and De Groot et al. (8) also found that the stimulus-induced release of vWF was dependent on the presence of extracellular Ca^{+}, and that release of vWF required an influx of extracellular Ca^{2+} (8).

The calcium-ionophore A-23187 is known for its ability to increase Ca^{2+} influx into cells. It is probably this property which enables it to induce the release both t-PA and vWF into the perfusate fluid in a rapid acute fashion. It has also been reported that the protein kinase C activator PMA stimulates the influx of Ca^{2+} into cultured endothelial cells (8) and releases vWF. In our perfusion system, PMA also released t-PA and vWF.

This release, though rapid, did not follow the usual time course of the stimulus-induced release reaction we see with other compounds, such as PAF or A-23187, but remained at a constant level for 15 min (Fig. 5).

The acute release of t-PA and vWF thus appear to be closely linked. Acute, transient release of the one is parallelled by acute, transient release of the other (e.g. as induced by PAF, bradykinin and A-23187), while PMA induces a constant release of both compounds. Also, DDAVP induces neither protein, while release of both proteins is insensitive to cycloheximide pretreatment and requires extracellular calcium. Moreover, the released amounts of both proteins are quantitatively correlated.

The present data do not allow to decide whether the close link between the two release processes is due to, for example, coupling of both to the same cellular receptor complexes, or due to a common cellular secretion mechanism, or - most likely - to a combination of both possibilities. The difference in the ratio of t-PA released over vWF released for different stimuli (compare e.g. PAF in Fig. 1 to A-23187 in Fig. 4) at least suggests that the two release processes are likely not identical.

The close link between the release of t-PA and that of vWF described here is also of interest in view of the linking of both processes in some subgroups of von Willebrand disease patients (see e.g. 33-37), where no acute release of t-PA (activity, refs. 33-35, and antigen, ref. 36) is found after stimulation.

As discussed previously (17), the acute release of t-PA, the production of endothelial cell-dependent relaxation factor(s) and the production of prostacyclin are closely linked in endothelial cells.
The acute release of vWF may also belong to this cluster of acute cellular endothelial reactions. From the data presented in this paper one can conclude that the mechanism of acute release of both t-PA and vWF is very similar and could involve the same cellular pathways.

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CHAPTER 5

THE INVOLVEMENT OF PHOSPHOLIPASES AND EICOSANOID METABOLISM IN THE ACUTE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR FROM PERFUSED RAT HINDLEGS

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1. Cellular mechanisms involved in the acute release from endothelial cells of tissue-type plasminogen activator (t-PA) were studied using a perfused rat hindlegs system.
2. Release of t-PA, which peaked at one min after adding a release-inducing compound to the perfusion medium, was induced by platelet activating factor (PAF), bradykinin, substance P, thrombin, carbachol, and A-23187.
3. Release of t-PA by the above-mentioned agents, was inhibited by mepacrine and by nor-dihydroguaiaretic acid.
4. PAF induced t-PA release was also inhibited by eicosatetraynoic acid, but not by oleic acid, linoleic acid, indomethacin or BW 755C.
5. PAF-induced release of t-PA was inhibited by the cytochrome P-450 mono-oxygenase inhibitors metyrapone, ketoconazole and SKF 525A.
6. Phospholipase A_2 did not induce any t-PA release, while (one type of) phospholipase C did.
7. PAF-induced von Willebrand factor (vWF) release was also similarly inhibited by the cytochrome P-450 mono-oxygenase inhibitor ketoconazole, while phospholipase C also induced vWF release.
8. Phorbol myristate acetate itself induced t-PA and vWF release, while subsequent release induced by PAF was reduced for t-PA but enhanced for vWF.
9. Release induced by PAF and bradykinin was not influenced by pretreatment with pertussis toxin.

INTRODUCTION

It is now recognized that the vascular endothelium is not simply a continuous monolayer of cells separating blood from tissues, but also a highly active metabolic tissue (see e.g. Vane et al., 1990). Among its functions are the synthesis and release of components involved in blood coagulation, haemostasis and fibrinolysis, such as von Willebrand factor (vWF), prostacyclin (PGI_2), endothelium-derived relaxing factor (EDRF), and tissue-type plasminogen activator (t-PA). Despite the importance of these components for vascular homeostasis, we still know very little about many aspects of the physiological and pharmacological regulation of these compounds. Because of our interest in the *in vivo* regulation of blood fibrinolytic activity, we have studied (Emeis, 1983; Emeis & Kluft, 1985; Tranquille & Emeis, 1988, 1989, 1990; Pruis & Emeis, 1990) cellular mechanisms involved in the acutely induced release of t-PA (and vWF; see Tranquille & Emeis, 1990) from endothelial cells (reviewed in Emeis, 1988). This process of acute stimulated secretion to a large extent determines the plasma level of t-PA (Emeis et al., 1990). Cytochrome P-450 mono-oxygenase inhibitors have been shown to inhibit the release of EDRF (Singer et al., 1984; MacDonald et al., 1986; Pinto et al., 1986, 1987; Förstermann et al., 1988; Rees et al., 1988), while certain phospholipases have been shown to induce release of EDRF and PGI_2 from aortic endothelial cells (De Nucci et al., 1988). As a sequel to the data reported above, and of previous observations made by ourselves (Emeis & Kluft, 1985; Tranquille & Emeis, 1988) on the effects of inhibitors of phospholipases and eicosanoid metabolism on t-PA release, this paper enlarges on the above observations, by the use of cytochrome P-450 inhibitors, phospholipase A_2 and C, pertussis toxin pretreatment, and a protein kinase C activator.

METHODS

Perfusion procedure.

Male Wistar rats (Broekman Institute, Someren, The Netherlands), weighing 220-300 g, were used. All experiments were performed under Nembutal anaesthesia (60 mg kg^{-1} i.p.). Rat hindleg perfusion was performed as described previously (Tranquille & Emeis, 1988). In brief: hindlegs were perfused through the aorta at constant flow (9-10 ml min^{-1}) with Tyrode's salt solution (composition in mM: NaCl 146, KCl 6, $CaCl_2$ 3, $MgCl_2$ 0.5, KH_2PO_4 0.3, $NaHCO_3$ 20, glucose 5.6), containing bovine serum albumin (0.1 mg ml^{-1}: Tyrode/BSA), pH = 7.5, at 37°C, and continuously gassed with 95% O_2/5% CO_2. Samples were obtained from a vena cava cannula. After a thirty-min wash-out period with Tyrode/BSA, a pre-sample was obtained. Subsequently, a release-inducing agent was added to the Tyrode/BSA and thirty-second sample blocks were obtained at 1 min

intervals for 5 min. Samples were rapidly cooled to 4 °C, centrifuged, mixed 10:1 with 0.5 M Tris.HCl, 1% Triton X-100 (pH = 7.5) and stored at -20 °C.
Unless indicated otherwise, inhibitors were added to the Tyrode/BSA during the last 20 min of the wash-out period, and were also present during the 5 min stimulation period.

Pertussis toxin pretreatment.
Pertussis toxin (50 $\mu g\ kg^{-1}$) was injected (i.v.) into rats 24 hours before perfusion (Ramkumar & Stiles, 1990). Control animals were injected with 0.5 ml saline.

Assay for t-PA.
The t-PA activity of perfusate samples was determined spectrophotometrically by the method of Verheijen et al. (1982), as described (Tranquille & Emeis, 1988). Human melanoma t-PA was used for calibration. Results will be expressed in Units ml^{-1}, one U of rat t-PA being equivalent in our assay to one IU of human t-PA, as defined by the International Standard of t-PA (batch 83/517). The detection limit of the assay was 0.03 U ml^{-1}.

ELISA determination for von Willebrand factor (vWF).
The presence of vWF in the perfusion samples was determined using an ELISA assay adapted from Ingerslev (1987) as detailed elsewhere (Tranquille & Emeis, 1990). Briefly, 96-well microtiter plates were coated with rabbit anti-human vWF immunoglobulin and left overnight at 4 °C. After rinsing the perfusate samples, 25 μl, and 75 μl of PBS-Tween buffer (pH 7.2) were added. Bound antigen was detected using peroxidase-conjugated rabbit immunoglobulins to human vWF. Absorbances were measured at 450 nm in a Titertek multiscan spectrophotometer. Rat pooled plasma in a range of 0.05 to 5.0% was used in each plate for calibration. The concentration of vWF will be expressed as Units, a hundred Units being equivalent to the amount of vWF present in one ml of pooled rat plasma. The detection limit of the assay was 0.1 U ml^{-1}.

Lactate dehydrogenase detection.
Lactate dehydrogenase (LDH) was measured using a single reagent system kit. 50 μl of each perfusion sample was added to 200 μl of the reconstituted reagent, containing lactate and nicotinamide adenine dinucleotide, in wells of a microtiter plate. Absorbances were measured at 340 nm in a Titertek multiscan spectrophotometer. The change in absorbances over a period of 90 minutes is expressed as LDH relative units.

MATERIALS

Platelet-activating factor (PAF) was obtained from Bachem (Bubendorf, Switzerland); Nembutal was from Sanofi (Paris, France). Rabbit anti-human vWF immunoglobulin and peroxidase-conjugated rabbit immunoglobulins to human vWF came from Dakopatts (Copenhagen, Denmark).

Bovine serum albumin (fraction V), substance P acetate, bradykinin triacetate, carbachol, mepacrine, diethylcarbamazine, nor-dihydroguaiaretic acid, acetylsalicylic acid, indomethacin, 4β-phorbol 12-myristate 13-acetate (PMA), cimetidine, 2-methyl-1,α-D1-3-pyridyl-1-propanon (metyrapone), pertussis toxin, phospholipase A_2 (PLA_2 from Naja naja and from porcine pancreas) and phospholipase C (PLC from Clostridium perfringens and from Bacillus cereus) were obtained from Sigma Chemical Co. (St. Louis, USA); LD-L-single reagent system for lactate dehydrogenase came from Sigma Diagnostics (St. Louis, USA); p-bromophenacylbromide from Aldrich Europe (Beerse, Belgium); bovine thrombin from Leo Pharmaceuticals (Copenhagen, Denmark); arachidonic acid, oleic acid and linoleic acid from Fluka (Buchs, Switzerland); A-23187 (free acid) from Boehringer Mannheim (Mannheim, FRG); and ketoconazole from Janssen (Beerse, Belgium). SKF 525A (proadifen) was a gift from Smith, Kline and French Ltd. (The Netherlands), BW 755C (3-amino-1[m-(trifluoromethyl)-phenyl]-2-pyrazoline) was a gift from the Wellcome Research Laboratories, Beckenham, US; 5,8,11,14-eicosatetraynoic acid (ETYA) was provided by Unilever Research Laboratories, Vlaardingen, The Netherlands. Materials used in the spectrophotometric PA assay have been detailed elsewhere (Verheijen et al., 1985); all other reagents used were of analytical grade.

Data analysis.

Data will be presented as mean ± standard deviation. Statistical significance of differences was established by one-way or two-way analysis of variance (ANOVA), followed by Bonferroni's modified t-test; or by Student's t-test.

RESULTS

Effect of phospholipase and lipoxygenase inhibitors.

In a previous paper we have shown that the release of t-PA as induced by platelet-activating factor (PAF) was inhibited by phospholipase inhibition, and by lipoxygenase inhibition (Emeis & Kluft, 1985). To see whether this inhibition profile was more generally applicable to compounds that induce t-PA release, the effects of the lipoxygenase inhibitor NDGA (70 μmol l^{-1}) and of the phospholipase inhibitor mepacrine (100 μmol l^{-1}) on t-PA release as induced by five other, unrelated, release inducing

compounds was studied (Table 1). These five compounds (i.e. bradykinin, substance P, thrombin, carbachol and the ionophore A-23187) all gave t-PA release with a time course similar to that induced by PAF (Fig. 1), though with different potencies (Fig. 1; Table 1). Inhibition by NDGA and mepacrine was nearly complete in all instances (Table 1).

Table 1. Effect of inhibitors on acute release of tissue-type plasminogen activator (t-PA) from perfused rat hindlegs.

All values obtained in the presence of inhibitor were significantly decreased compared to control values by one-way ANOVA (all $p < 0.01$).

Stimulating compound (concentration)	t-PA released (U ml^{-1}) Inhibitor (concentration)*		
	Controls	NDGA (70 $\mu mol\ l^{-1}$)	Mepacrine (100 $\mu mol\ l^{-1}$)
Bradykinin † (0.8 $\mu mol\ l^{-1}$)	2.15 ± 0.40 (10)	0.26 ± 0.01 (7)	0.15 ± 0.04 (4)
Substance P (0.8 $\mu mol\ l^{-1}$)	1.36 ± 0.24 (8)	0.24 ± 0.09 (5)	0.15 ± 0.06 (4)
Thrombin (40 $nmol\ l^{-1}$)	0.44 ± 0.10 (7)	0.12 ± 0.08 (4)	0.10 ± 0.03 (4)
Carbachol (25 $\mu mol\ l^{-1}$)	0.36 ± 0.09 (10)	0.10 ± 0.04 (8)	0.10 ± 0.06 (6)
PAF ‡ (20 $nmol\ l^{-1}$)	2.74 ± 0.65 (12)	0.38 ± 0.34 (7)	0.53 ± 0.48 (5)
A-23187 (2 $\mu mol\ l^{-1}$)	2.36 ± 0.34 (5)	0.79 ± 0.08 (6)	0.68 ± 0.43 (6)

* Inhibitors were added to the Tyrode/BSA for 20 min prior to stimulation, and during the 5 min stimulation period.

† Mean ± sd (number of determinations) of maximal concentration of t-PA obtained during a 5 min stimulation period.

‡ Data from Emeis & Kluft (1985).

Inhibition of the PAF-induced t-PA release by a range of inhibitors related to eicosanoid metabolism is shown in Table 2. The release was not significantly affected by cyclo-oxygenase inhibitors (aspirin, indomethacin) and by BW 755C, but was significantly decreased by p-bromophenacylbromide and eicosatetraynoic acid (ETYA). The effect of ETYA was not due to an aspecific detergent-like effect, as oleic acid and linoleic acid had no significant effect on release. However, arachidonic acid, like ETYA, was a strong inhibitor (Table 2).

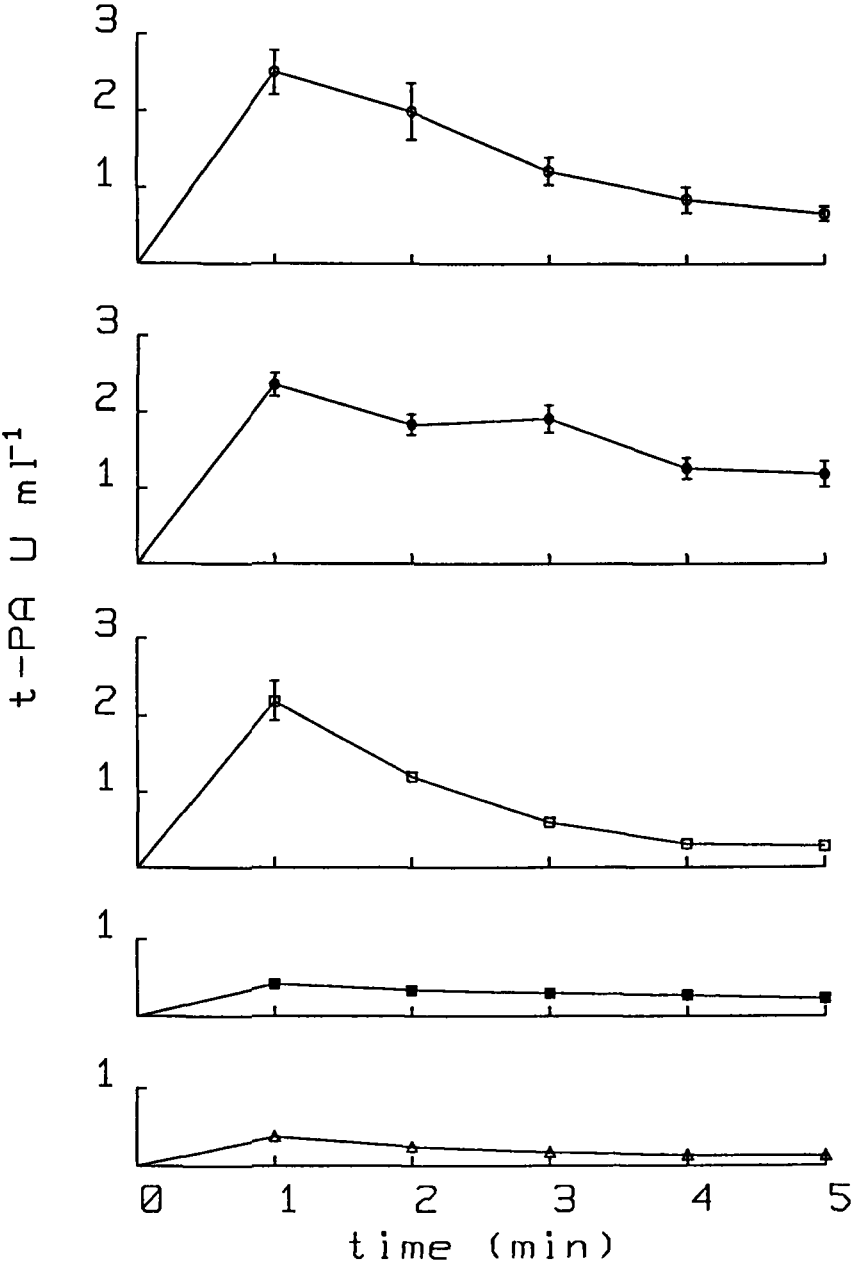

Fig. 1. Time course of t-PA concentrations in the perfusate during a five-min stimulation period. Thirty-second sample blocks were obtained at one-min intervals from a vena cava outflow cannula. Data are shown as means (n = 5-7) with vertical lines indicating sd. The release of t-PA was induced by: 5 nmol l^{-1} PAF (o); 2 μmol l^{-1} A-23187 (•); 0.8 μmol l^{-1} bradykinin (□); 25 μmol l^{-1} carbachol (■); and 40 nmol l^{-1} thrombin (▵).

Table 2. Effect of inhibitors on tissue-type plasminogen activator (t-PA) release in rat perfused hindlegs induced by platelet-activating factor (5 nmol l^{-1}).

Inhibitor	Concentration (μmol l^{-1})	Number of experiments	t-PA released (U ml^{-1})
-	-	13	1.65 ± 0.70*
acetylsalicylic acid	30	5	1.85 ± 0.40
indomethacin	30	5	1.15 ± 0.20
BW 755C	200	3	1.90 ± 0.60
p-bromophenacylbromide	100	3	0.50 ± 0.10 ‡
eicosatetraynoic acid †	33	3	0.65 ± 0.05 ‡
oleic acid †	33	3	2.20 ± 0.50
linoleic acid †	33	3	1.25 ± 0.30
arachidonic acid †	33	7	0.20 ± 0.15 ‡

* All data shown are mean ± s.d. of maximal t-PA concentrations obtained during the five min stimulation period.

† Fatty acids were present in the Tyrode/BSA for 10 min, followed by a five-min wash-out period, prior to PAF stimulation.

‡ Significantly different from controls by one-way ANOVA, followed by Bonferroni's modified t-test ($p < 0.01$).

Effect of cytochrome P-450 mono-oxygenase inhibitors.

As the above data suggested that epoxyeicosatrienoic acid formation could be involved (compare Pritchard et al., 1990), we next looked at the effects of inhibitors of cytochrome P-450-mediated mono-oxygenation (Table 3) on PAF-induced t-PA release. At 0.5 mmol l^{-1}, metyrapone inhibited by 40%, and by 90% at 1 mmol l^{-1}. SKF 525A inhibited by one-third at 26 μmol l^{-1}; at 52 μmol l^{-1}, however, a blockade of the perfusate flow through the hindleg occurred, which made interpretation of the SKF 525A data difficult. Ketoconazole dose-dependently inhibited t-PA release (Table 3). Separate experiments showed that ketoconazole also reduced vWF release (solvent controls: 1.80 ± 0.64 U ml^{-1}; ketoconazole at 100 μmol l^{-1}: 0.65 ± 0.28 U ml^{-1}, i.e. a 64% inhibition of vWF release). The induction of release by 0.8 μmol l^{-1} bradykinin was also inhibited by ketoconazole at 100 μmol l^{-1}: t-PA release was reduced by 75% and vWF release by 40%. The H_2-receptor blocker, cimetidine (1 mmol l^{-1}), which also has an inhibitory effect on cytochrome P-450 mono-oxygenases in some instances (Bast et al., 1984; Förstermann et al., 1988), had no inhibitory effect on the acute release of t-PA or vWF induced by bradykinin and PAF in our system (data not shown).

Table 3. Effects of cytochrome P-450 inhibitors on the induced release of tissue-type plasminogen activator (t-PA) by platelet-activating factor in perfused rat hindlegs.

Inhibitor (concentration)	Number of experiments	t-PA released (U ml^{-1})
-	5	2.96 ± 0.39*
Metyrapone (0.5 mmol l^{-1})	8	1.70 ± 0.58 †
Metyrapone (1 mmol l^{-1})	4	0.29 ± 0.45 †
SKF 525A (26 μmol l^{-1})	4	1.92 ± 0.65
SKF 525A (52 μmol l^{-1})	2	no perfusate flow
Solvent controls	4	2.52 ± 0.18
Ketoconazole (25 μmol l^{-1})	4	3.10 ± 0.44
Ketoconazole (50 μmol l^{-1})	4	1.12 ± 0.23 †
Ketoconazole (100 μmol l^{-1})	4	0.43 ± 0.11 †

* Mean ± s.d. of peak levels of t-PA release induced by PAF (20 nmol l^{-1}) during a five-min stimulation period.

† Significantly different from control values by one-way ANOVA, followed by Bonferroni's modified t-test ($p < 0.01$).

Effect of externally applied phospholipases.

Having studied the effects of fatty acid metabolism on the acute release of t-PA, it was decided to see whether the release of t-PA and vWF may be induced directly by a phospholipase, as described by De Nucci et al. (1988).

PLA_2 from two different sources (Naja naja venom and porcine pancreas) and PLC from two different sources (B. cereus and C. perfringens) were investigated. Each enzyme was perfused through the rat hindleg for 10 mins with sample collections taken every minute. After a five minute washout period PAF (5 nmol l^{-1}) was perfused to check the viability of the perfusion system.

Both types of PLA_2 were perfused at concentrations of 20 mU ml^{-1} and 40 mU ml^{-1}. At none of these doses was any acute release of t-PA or vWF observed over the 10 minutes perfusion sampling period. The PAF stimulation following the PLA_2 perfusion was found to be normal.

Similar observations were made when PLC (B. cereus) was perfused. This PLC did not release any t-PA or vWF and had no effect on the PAF stimulation following it.

However, PLC (C. perfringens) did release both t-PA and vWF dose-dependently,

starting at 5 mU ml^{-1} (see Fig. 2). At 20 mU ml^{-1} concentration PLC blocked flow through the system after 9 mins. At 40 mU ml^{-1} this blockage occurred after 6 mins of PLC perfusion. The PLC perfusate samples were tested for the presence of lactate dehydrogenase (LDH). In the samples containing peak amounts of t-PA and vWF, LDH was found to be close to baseline levels (Fig. 3). LDH levels increased during the perfusion period but were not correlated to the release of t-PA and vWF.

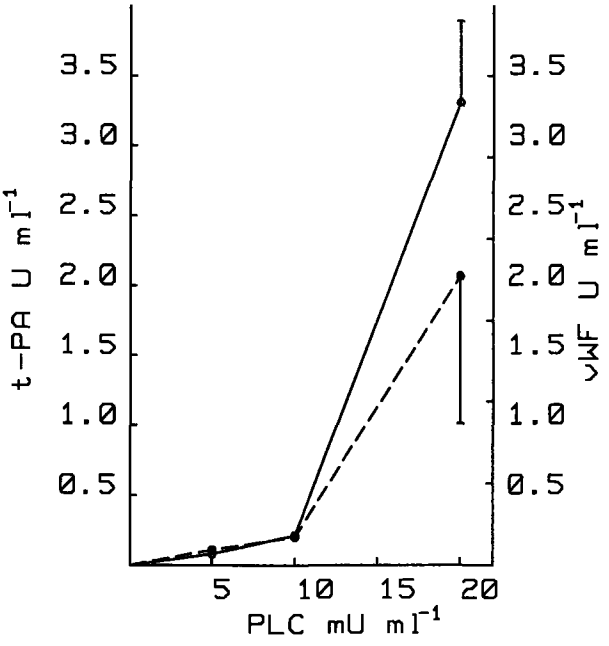

Fig. 2. Dose-dependent release of t-PA (o) and vWF (•) induced by phospholipase C from C. perfringens (expressed as mU ml^{-1} of perfusion fluid). Data shown at maximal release point (n = 1 for 5.10 mU ml^{-1} and n = 3 for 20 mU ml^{-1}).

Effect of PMA on t-PA and vWF release.

As phospholipase C activity results in the formation of, among others, diacylglycol (DAG), and as protein kinase C (PKC) activation may have a role in acute release, the DAG analogue, PMA, which is also a PKC activator, was studied. PMA (at 100 nmol/l^{-1}) induced, after a lag period of 2 min, a t-PA and vWF release of about 0.3 U ml^{-1} which remained stable for 15 min (Tranquille & Emeis, 1990). After preperfusion for 15 min

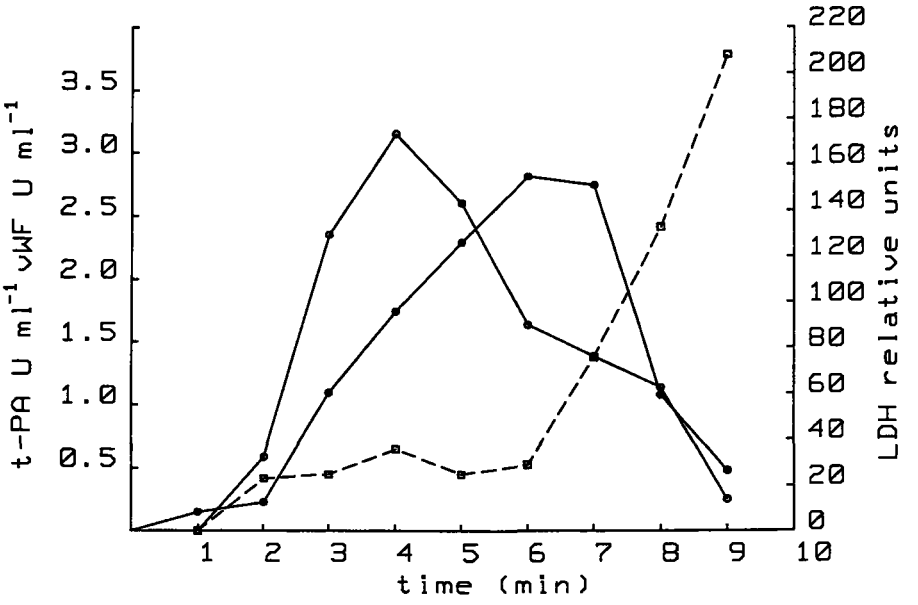

Fig. 3. Time-course of t-PA, vWF, and LDH release induced by phospholipase C (C. perfringens, 20 mU ml^{-1}) in perfused rat hindlegs. Data given for one typical experiment, t-PA release (U ml^{-1};o), vWF release (U ml^{-1};•), and LDH (relative units of absorption at 340 nm; □).

with PMA, PAF induced significantly less t-PA release, compared to control preparations preperfused with solvent (Fig. 4; Table 4). Similar results were obtained when A-23187 was used to induce t-PA release (Table 4). However, in contrast, after preperfusion for 15 min with PMA, PAF and A-23187 induced significantly more vWF release, compared to control preparations preperfused with solvent (Fig. 4).

Effect of pertussis toxin pretreatment on acute release.

The effect of pertussis toxin pretreatment on acute release was next investigated. No difference in the acute release of t-PA or vWF induced by both bradykinin and PAF, was seen between a pertussis toxin pretreated group and a saline control group (data not shown).

DISCUSSION

Endothelial stimulation by a wide variety of agonists (see e.g. Fig. 1 and Table 1) results in the rapid release of t-PA (review by Emeis, 1988), and of vWF (Tranquille & Emeis, 1990). We previously suggested (Emeis & Kluft, 1985) that fatty acid liberation and

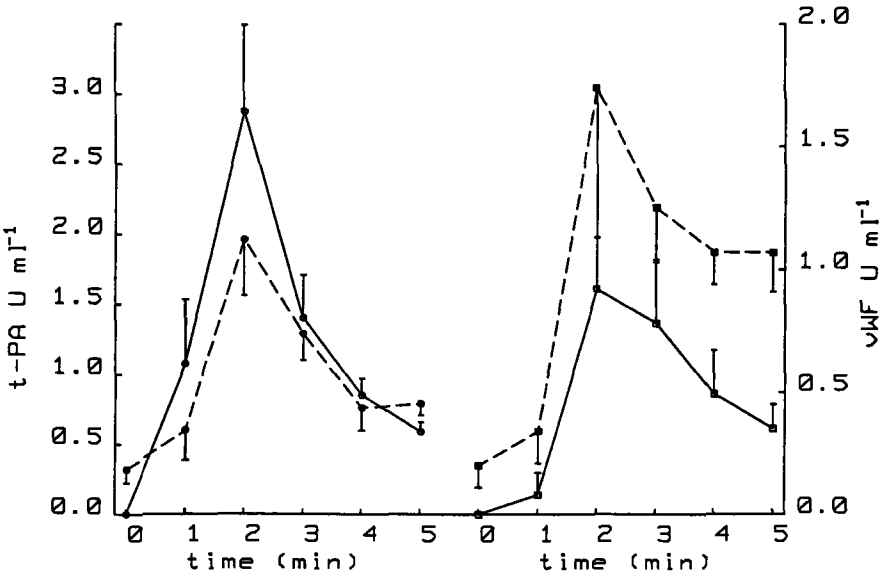

Fig. 4. Time course of t-PA and vWF release during a 5-min stimulation period with PAF (5 nmol l^{-1}). Comparison of controls perfused with ethanol (0.1%) t-PA (o), vWF (□) and those conducted in the presence of PMA t-PA (•) vWF (■) which had been perfused through the system for 15 min prior to stimulation. The release of t-PA is significantly decreased, while that of vWF is significantly increased after PMA-pretreatment compared to ethanol-pretreatment. Data are shown as means (n = 4) with vertical lines indicating sd (compare Table 4).

Table 4. Effect of pretreatment with phorbol myristate acetate (PMA) on release of tissue-type plasminogen activator (t-PA) from perfused rat hindlegs induced by platelet-activating factor (PAF) or A-23187.

PMA-pretreatment significantly reduced t-PA release (two-way ANOVA; $p < 0.005$).

Pretreatment*	t-PA release (U ml^{-1}) induced by	
	PAF (5 nmol l^{-1})	A-23187 (2 μmol l^{-1})
solvent	2.88 ± 0.62 (4) †	3.0; 2.65 (2)
PMA	1.96 ± 0.50 (4)	1.60 ± 0.39 (4)

* After a 30 min preperfusion with Tyrode/BSA, rat hindlegs were perfused for 15 min with solvent (0.1% ethanol) or PMA (100 nmol l^{-1}), followed by stimulation with PAF or A-23187 in the continued presence of solvent or PMA.

† Mean ± sd (number of determinations) of maximal t-PA concentration in effluent fractions obtained at 1 min intervals during a 5 min stimulation period.

subsequent lipoxygenation was a prerequisite for PAF-induced release to occur, this suggestion being based on studies using phospholipase and lipoxygenase inhibitors. In the present study, applying similar inhibitors to inhibit release induced by other compounds, we obtained the same result, i.e. a reduction of t-PA release. The use of a larger number of inhibitors, however, including the combined lipoxygenase-cyclooxygenase inhibitor BW 755C and three inhibitors of cytochrome P-450-mediated mono-oxygenation, i.e. ketoconazole, metyrapone and SKF 525A, now lead us to suggest that mono-oxygenases rather than lipoxygenases are likely to be involved in the release of t-PA and vWF. This suggestion would concur with a variety of reported data on endothelial cell metabolism.

Endothelial cells (EC) contain cytochromes P-450 (e.g. Baird et al., 1980; Dees et al., 1982; Abraham et al., 1985; Forkert et al., 1989), and a cytochrome P-450-mediated mono-oxygenation system (e.g. Johnson et al., 1985; Pinto et al., 1986, 1987; Revtyak et al., 1988). The synthesis of epoxyeicosatrienoic acids (EETs) in EC is inhibited by NDGA, ETYA and SKF 525A, but not by cyclooxygenase inhibitors or by BW 755C (Pritchard et al., 1990; compare Bednar et al., 1984), an inhibition profile identical to that found in the present study for release. EET synthesis is stimulated by histamine and thrombin (Revtyak et al., 1988), which also induce release of t-PA and vWF from EC, while EETs have been shown to induce cellular secretion in a variety of cells (reviewed by Fitzpatrick & Murphy, 1989), though such a direct effect of EETs on secretion has not yet been studied in EC.

The slight, non-significant, inhibitory effect of indomethacin on release might also be ascribed to an effect on mono-oxygenation, as indomethacin has a weak inhibitory effect on this reaction (Capdevila et al., 1988). A role for cyclooxygenase in the induction of t-PA release is unlikely, as aspirin did not inhibit release and neither did BW 755C, a combined cyclooxygenase and lipoxygenase inhibitor.

Others have demonstrated a similar inhibitory profile for the induction of endothelial cell-dependent relaxation, giving rise to the speculation that EDRF was a product of arachidonic acid metabolism (Singer et al., 1984; Pinto et al., 1987). However, it is now known that EDRF is nitric oxide or a closely related compound. The effects of lipoxygenase- and phospholipase-inhibitors on EDRF must thus be ascribed to either an inhibitory effect on cGMP formation (Förstermann et al., 1988; Johns & Peach, 1988; Johns et al., 1988) or on the induction of EDRF formation (Förstermann et al., 1988). As we have found in separate experiments (Tranquille & Emeis, ms submitted) that induction of cGMP synthesis by e.g. sodium nitroprusside or atrial natriuretic factor, strongly reduced the release of t-PA and vWF, the present data can not be ascribed to an effect on cGMP.

The suggestion about a role of EETs in the release of t-PA and vWF leaves the inhibitory effect of arachidonic acid unexplained. Possibly, arachidonic acid in EC gave

rise to metabolites that inhibited release, which would explain the rapid down-regulation of release that occurs over a five-min period (Fig. 1). If so, these metabolites are not cyclooxygenase products, as aspirin and indomethacin did not prevent the inhibitory effect of arachidonic acid (unpublished data). As arachidonic acid alone does not induce (Surichamorn et al., 1990) the calcium influx necessary for release to occur (Pruis & Emeis, 1990; Tranquille & Emeis, ms submitted) the inhibitory effects might then have superseded any other effects.

Exogenous addition of PLC (from C. perfringens) induced the release of t-PA and vWF dose-dependently from the perfused hindleg (Fig. 2). De Nucci et al. (1988) have shown that PLC induces the release of EDRF and PGI_2 from cultured bovine aortic endothelial cells, but that PLA_2 only induced the release of PGI_2. In our model PLA_2 did not induce the release of either t-PA or vWF and it did not affect the induced release of either of them by PAF. All these data would fit in our hypothesis (Tranquille & Emeis, ms submitted) that, like the release of EDRF (Lückhoff et al., 1988; Adams et al., 1989), the release of t-PA and vWF requires influx of extracellular calcium ions, while PGI_2 synthesis would require only an increase in intracellular calcium (Hallam et al., 1988). As described by Surichamorn et al. (1990) PLA_2 does not induce calcium influx.

The release of t-PA and vWF by PLC may involve a specific PLC-induced pathway of transduction in the cell, i.e. the formation of diacylglycerol and inositol phosphates (Berridge, 1987; Nishizuka, 1986; Berridge & Irvine, 1984). Perfusion of rat hindlegs with PMA, a diacylglycerol analogue, which activates protein kinase C (Blumberg et al., 1984) resulted in a continuous significant, but small increase in t-PA and vWF release (Tranquille & Emeis, 1990). However, activation of protein kinase C alone does not appear sufficient to induce the full-scale t-PA or vWF release reaction, possibly because of the absence of calcium influx. PLC (C. perfringens) induced more release than did PMA, and with a different pattern in time (Fig. 3) than PMA, more closely resembling the release pattern obtained with the other, calcium flux-inducing, compounds (Fig. 1). Treatment with PLC (C. perfringens) may result in calcium influx, giving rise to t-PA and vWF (this paper) and EDRF (De Nucci et al., 1988) release. After a 15 min pretreatment with PMA, the release of t-PA induced by PAF or A-23187 was reduced by some 40%. The decrease, after PMA, of PAF- or A-23187-induced t-PA release could be due to feed-back inhibition by activated protein kinase C, possibly of a G-protein involved in t-PA release, as suggested by De Nucci et al. (1988) for the inhibition of EDRF release. The release of vWF by PAF after PMA pretreatment was significantly increased (Fig. 4), so that receptor down-regulation is not likely to be involved in the decreased release of t-PA after PMA. In agreement with this, the (receptor-independent) release induced by the calcium ionophore A-23187 was similarly affected by PMA (Table 4). The results observed with pertussis toxin pretreatment, where no change in t-PA or vWF levels (induced or baseline) in the perfusate samples (or in rat plasma samples,

unpublished observation), was detected, imply that a pertussis toxin-sensitive G-protein is not likely to mediate the intracellular signal resulting from stimulation by bradykinin and PAF. In addition, the actual mechanism of release does not seem to involve a pertussis toxin-sensitive G-protein.

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CHAPTER 6

ON THE ROLE OF CALCIUM IN THE ACUTE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND VON WILLEBRAND FACTOR FROM THE RAT PERFUSED HINDLEG REGION

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ABSTRACT

The involvement of calcium in the release of tissue-type plasminogen activator (t-PA) and von Willebrand Factor (vWF) from vascular endothelial cells was studied using a rat hindleg perfusion system. By adding either platelet-activating factor or bradykinin to the perfusing Tyrode solution, a rapid release of t-PA and vWF was induced. Extracellular calcium was essential for the acute release of both glycoproteins as this release was totally abolished in the presence of EGTA. The calcium ionophore A-23187 induced the release of both proteins. The use of the calcium L-type channel blockers, verapamil and diltiazem, and the calcium channel agonist BAY K-8644, did not provide any evidence for the involvement of endothelial voltage-operated calcium channels. Trifluoperazine, a calmodulin antagonist, significantly inhibited the induced release of t-PA and vWF. However, calmidazolium, another calmodulin antagonist, itself induced the release of both t-PA and vWF. Lanthanum chloride inhibited the induced release of t-PA but not that of vWF. Our results suggest that Ca^{++} influx is essential for the release of t-PA and vWF from the perfused rat hindleg.

INTRODUCTION

The vascular endothelium plays a very important role in the maintenance of vascular homeostasis. Endothelial cells are now known to synthesize and secrete both activators and inhibitors of the coagulation system and of the fibrinolytic system, and mediators such as prostacyclin (PGI_2) and endothelium derived relaxing factor (EDRF) that are

involved in the adhesion and aggregation of blood platelets. Two of the many compounds that can be secreted from endothelial cells are tissue-type plasminogen activator (t-PA), the key enzyme in the fibrinolytic system necessary to activate plasminogen to plasmin (8), and von Willebrand Factor (vWF) which is necessary for platelet adhesion to the subendothelium when vascular injury occurs (5).

The precise mechanism involved in the release of these two proteins from endothelial cells is still largely unknown. In past experiments, using our *ex vivo* model of the rat perfused hindleg (7), we have investigated various pathways involving cytochrome P-450 mono-oxygenases, phospholipases, arachidonic acid cascade products, cyclic nucleotides, protein kinase-C and protein synthesis (9,22,23,24, and unpublished observations) in relation to the acute release of t-PA and vWF from the endothelium. In the studies mentioned above we had conducted some preliminary experiments (9,17,24) suggesting that extracellular calcium was required for the acute release of both t-PA and vWF. Loesberg et al. (14) and De Groot et al. (4) had showed that in cultured human endothelial cells the release of vWF also requires extracellular Ca^{++}, as does the release of protein S from those cells (21). Therefore, a more detailed study was performed to investigate the role of calcium in the acute release of the two proteins t-PA and vWF *ex vivo*.

These experiments involved the use of the calcium ionophore A-23187, the calcium channel agonist BAY K-8644, calcium entry blockers such as lanthanum chloride, verapamil and diltiazem, the "intracellular calcium antagonist" TMB-8, and the calmodulin antagonists calmidazolium and trifluoperazine (TFP) (7,20).

METHODS

The following procedures are described only briefly below as they have been described in detail previously (e.g. 22,24).

Perfused rat hindleg system.

Male Wistar rats (220-300 g; Nembutal anaesthesia) were used. The hindlegs were perfused via the aorta at constant flow (10 ml/min) with oxygenated Tyrodes' balanced salt solution, containing bovine serum albumin (BSA) (0.1 mg/ml), at 37°C.

Effluent samples were collected from a vena cava outflow cannula. After a 30 min wash-out period, release-inducing compounds were perfused for 5 min each. Samples were collected at 1 min intervals for 30 seconds, centrifuged and either assayed immediately or stored at -20°C.

Experimental procedure.

Two compounds were used to stimulate release in each experimental animal; first bradykinin (0.8 μM) was perfused through the system for 5 min, followed by a 5 min wash-out period before platelet-activating factor (PAF; 5 nM) was perfused for 5 min. In experiments involving antagonists the drug under investigation was added to the Tyrode/BSA solution after 10 or 20 min and perfused for 20 (or 10) min before, and during, the period of stimulating release.

In the experiments involving the absence of Ca^{++} from the perfusion solution (and those using in addition 1 mM EGTA in the perfusion solution) the time scale used was different to that mentioned above. In these experiments Ca^{++} was omitted from the perfusates starting at 0, 2, 4, 6, 8 and 10 min before stimulation with PAF (5 nM) in the continued absence of Ca^{++} for 5 min. After a further 5 min wash-out without Ca^{++} PAF (5 nM) was again perfused through the system but this time in a normal Ca^{++}-containing Tyrode perfusion solution (and in the absence of EGTA).

Spectrophotometric plasminogen activator assay.

The PA activity of the perfusate samples was determined by the indirect spectrophotometric rate assay described by Verheijen et al. (26). The PA activity was determined to be t-PA activity as detailed previously (9,22).

Dilutions of human melanoma t-PA were run in each plate for calibration, and the activity of the samples will be expressed in Units, one Unit (of rat t-PA) being equivalent in our assay to one International Unit of human t-PA.

ELISA assay for rat von Willebrand Factor (24).

Microtiter plates were coated with rabbit anti-human von Willebrand Factor Ig and incubated with rat hindleg perfusate samples (25 μl). Bound antigen was quantitated using peroxidase-conjugated rabbit anti-human von Willebrand Factor Ig. Diluted pooled rat plasma (0.05-5% v/v) was used as a standard. The concentration of vWF will be expressed as Units, a hundred Units being equivalent to the amount of vWF present in one ml of pooled rat plasma.

MATERIALS

Platelet-activating factor (PAF) was obtained from Bachem (Bubendorf, Switzerland). Nembutal was from Sanofi (Paris, France). Bradykinin, bovine serum albumin (fraction V), mepacrine, nordihydroguaiaretic acid (NDGA), trifluoperazine (dihydrochloride), lanthanum chloride, verapamil (±), and diltiazem were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calmidazolium came from Janssen Pharmaceutica (Beerse,

Belgium). BAY K-8644 came from Research Biochemicals Inc. (Natick, MA, U.S.A.). A-23187 (free acid) came from Boehringer (Mannheim, FRG). 8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) and ethylenebis (oxyethylene nitrilo) tetraacetic acid (EGTA) came from the Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.). Rabbit anti-human von Willebrand Factor Ig and peroxidase-conjugated rabbit anti-human von Willebrand Factor Ig came from Dakopatts (Copenhagen, Denmark). Materials used in the spectrophotometric PA assay have been detailed elsewhere (26). All other reagents used were of analytical grade.

Data analysis.

Data will be presented as mean ± standard deviation. Statistical significance of differences was established by one-way or two-way analysis of variance (ANOVA), followed by Bonferroni's modified t-test; or by Student's t-test.

RESULTS

The induced release of t-PA by A-23187.

In our experimental model of the perfused hindleg we could not detect any baseline level of t-PA release in the effluent samples. The addition of a release-inducing agent such as bradykinin or PAF to the perfusion medium resulted in a rapid transient release of t-PA activity, peaking within the first two min of stimulation and then gradually decreasing over the next 3 min (22).

The calcium ionophore A-23187 (2 μM) was tested for induction of t-PA release in our perfusion model. A-23187 did induce t-PA release with a time-course similar to that described previously for the other release-inducing agents such as bradykinin and PAF (24). Release required the presence of Ca^{++} in the Tyrode/BSA solution (Table 1), and was significantly inhibited by the lipoxygenase inhibitor NGDA and by the phospholipase inhibitor mepacrine, similar to data shown previously for PAF-induced t-PA release (9).

The effects of calcium depletion on t-PA release.

Experiments were conducted to investigate the requirement of Ca^{++} for the acute release of t-PA in our system.

In the first series of experiments, the perfusion solution was changed to a nominally Ca^{++}-free Tyrode solution and perfused through the rat hindleg for timed intervals of 0, 2, 4, 6, 8 and 10 min before stimulation with PAF (5 nM, which was also diluted in the same Ca^{++}-free solution). The induced release of t-PA by PAF was decreased with the depletion of calcium. This reduction was found to be time-dependent: the longer the calcium was depleted, the less t-PA was released by PAF (see Fig. 1a).

Table 1. Effect of inhibitors on acute t-PA release induced by A-23187 (2 μM).

All values obtained in the presence of inhibitor were significantly decreased compared to control values by one-way ANOVA ($p < 0.01$).

	t-PA release (U/ml)	Number of experiments
Controls (A-23187 2 μM only)	2.36 ± 0.34[+]	5
No calcium + EDTA (1 mM)*	< 0.03	3
NDGA (70 μM)*	0.79 ± 0.08	6
Mepacrine (100 μM)*	0.68 ± 0.43	6

* Inhibitors added to the Tyrode/BSA solution for 20 min prior to stimulation, and during the 5 min stimulation period.

[+] Mean ± s.d. (peak values of 5 min perfusion periods).

Addition of the chelating agent EGTA (1 mM) to the Ca^{++}-free Tyrode solution reduced the release of t-PA by PAF faster (Fig. 1a). The presence of EGTA in the perfusion solution completely inhibited any t-PA release induced by PAF within 4 min.

The re-addition of calcium-containing Tyrode solution and PAF (5 nM) to the perfusion experiment after depletion of calcium for 10, 12, 14, 16, 18 or 20 min, resulted in the gradual return of t-PA release, as seen in Figure 1b. This release, however, never attained the 'normal control' levels (approx 3 U/ml). The highest values were recorded in those experiments where Ca^{++} had been depleted for 14 to 16 min before the second PAF stimulation in normal Ca^{++}-containing Tyrode solution was conducted. This observation was found to be similar in experiments done both in the presence and absence of EGTA (1 mM) from the Ca^{++}-free solution (Fig. 1b).

The addition of the inorganic Ca^{++} entry blocker, lanthanum chloride ($LaCl_3$) (20 μM, perfused for 10 min prior to stimulation) partially reduced the induced t-PA release by both bradykinin (0.8 μM) and PAF (5 nM) (Table 2). At 200 μM (perfused for 10 min prior to stimulation) $LaCl_3$ reduced the t-PA release induced by bradykinin and PAF much more strongly (Table 2). Control experiments showed that this concentration of $LaCl_3$ did not inhibit t-PA activity significantly.

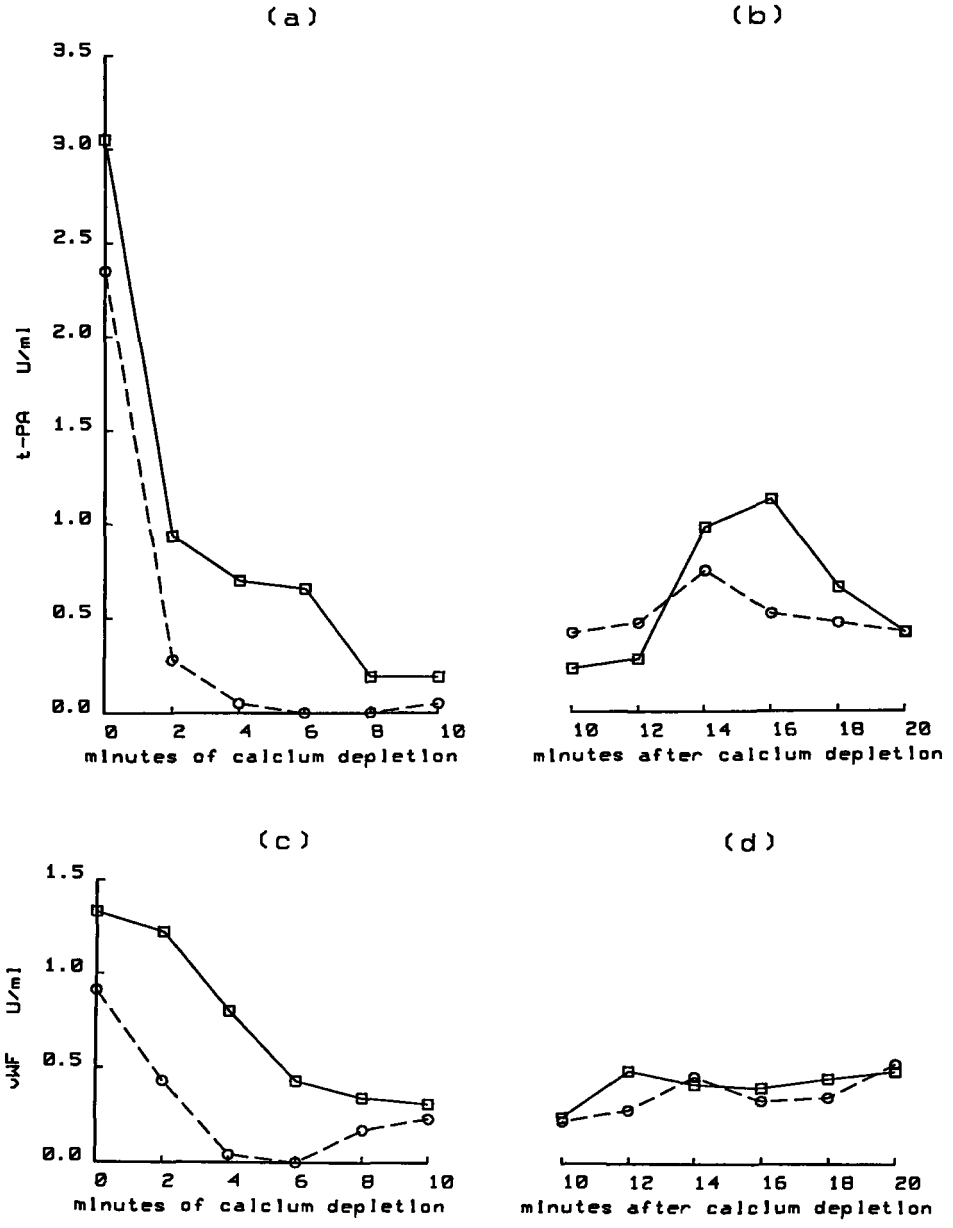

Fig. 1. The effect of depletion of extracellular calcium on the release of t-PA (a,b) and vWF (c,d). Time in minutes on the abscissa indicates the time of calcium depletion prior to PAF stimulation in (a) and (c), and the time in minutes after calcium depletion prior to a second PAF stimulation in calcium-containing Tyrode solution in (b) and (d). Calcium depletion was obtained by either deleting calcium from the Tyrode's perfusion solution (□-□) or by deleting calcium and adding 1 mM EDTA (o-o). Data shown are peak points of t-PA (a and b) and vWF (c and d) released. Each point represents a single experiment.

Table 2. Effects of lanthanium chloride on t-PA release.

	t-PA (U/ml) release induced by bradykinin (0.8 μM)	PAF (5 nM)	Number of experiments
Control	1.68 ± 0.27*	2.86 ± 0.39	7
$LaCl_3$ (20 μM)	1.36, 0.80	2.48, 1.54	2
$LaCl_3$ (200 μM)	0.40 ± 0.11**	0.26 ± 0.16**	4

* Mean ± s.d. (peak values of 5 min perfusion periods).

** Significantly different from control values (Student t-test; $p < 0.01$).

A few perfusion experiments were done in the presence of increased Ca^{++} concentration. Increasing the calcium concentration in the Tyrode/BSA perfusion solution from 3 mM to 9 mM did not enhance the acute release of t-PA (data not shown).

The effects of calcium channel agonists and antagonists on t-PA release.

To investigate the effects of calcium channel antagonists in our system we conducted experiments using two different L-type Ca^{++} channel blockers: verapamil (10 μM), a phenylalkylamine, and diltiazem (20 μM), a benzothiazepine compound. Both of these channel blockers were perfused for 10 min before stimulation with bradykinin and PAF. Neither of them induced any detectable t-PA release themselves.

Verapamil and diltiazem had no significant effect on t-PA release induced by bradykinin (Table 3). However, both verapamil and diltiazem reduced PAF-reduced t-PA release by 30-50% (Table 3) as we described previously for endothelin-3 induced t-PA release (17).

The calcium agonist BAY K-8644 (at a 1 μM; ref. 10) did not induce any t-PA release. It reduced the release of t-PA induced by bradykinin by 30% but had no effect on the release induced by PAF (Table 3).

The effects of calmodulin antagonists and of TMB-8 on t-PA release.

The calmodulin antagonist TFP significantly inhibited (at 50 μM) the release of t-PA induced by both bradykinin and PAF (Table 4). TFP itself did not induce any t-PA release. In contrast, the other calmodulin inhibitor tested, calmidazolium, itself induced - at 10 μM - release of t-PA. The amount induced by calmidazolium (1.67 ± 0.04 U/ml; n = 4; at 10 μM) was similar to that induced by 0.8 μM bradykinin (1.68 ± 0.27 U/ml). The time-course of calmidazolium-induced t-PA release was similar to the time-course

Table 3. Effects of a calcium channel agonist and antagonists on t-PA release.

	t-PA (U/ml) release induced by bradykinin (0.8 μM)	PAF (5 nM)	Number of experiments
Control	1.68 ± 0.27*	2.86 ± 0.39	7
Verapamil (10 μM)	1.24 ± 0.39	2.08 ± 0.56^{++}	4
Diltiazem (20 μM)	1.73 ± 0.46	1.53 ± 0.60^{++}	3
BAY K-8644 (1 μM)	1.19 ± 0.38^{+}	2.54 ± 0.76	4

* Mean ± s.d. (peak values of 5 min perfusion periods).
$^{+}$ Significantly different from control values using student t-test ($p < 0.05$).
$^{++}$ Significantly different from control values by one-way ANOVA ($p < 0.01$).

of release as induced by other agents, peak levels being found at two minutes. Calmidazolium also - again in contrast to TFP - greatly reduced flow rates during stimulation with both bradykinin (mean flow rate 37% of that observed in the absence of calmidazolium) and PAF (mean flow rate 24% of controls; see Table 4).

Table 4. Effects of the calmodulin antagonists on t-PA release.

Compound (concentration)	t-PA (U/ml) release induced by bradykinin (0.8 μM)	flow rate (% of control)	t-PA (U/ml) release induced by PAF (5 nM)	flow rate (% of control)	Number of experiments
Controls	1.68 ± 0.27*	100^{+}	2.86 ± 0.39	100	7
TFP (50 μM)	0.29 ± 0.07	75	0.08 ± 0.09	47	3
Calmidazolium (10 μM)	0.89 ± 0.22	37	0.29 ± 0.21	24	4
TMB-8 (70 μM)	1.51 ± 0.53	45	0.75 ± 0.32	22	6
Reverse TMB-8 (70 μM)^{x}	0.09, 0.05	12	1.92, 2.16	54	2

* Mean ± s.d. (peak values of 5 min perfusion periods).
$^{+}$ Mean value.
^{x} Reverse stimulation. First-PAF, and second-bradykinin (see text for explanation).

The 'intracellular Ca^{++} antagonist' TMB-8 did not induce any t-PA release itself and had no significant effect on the induced t-PA release by bradykinin but significantly reduced the t-PA release induced by PAF (Table 4). TMB-8 also caused a great reduction in the flow rate. As the flow effects became more apparent during the second stimulation, reverse experiments were conducted using TMB-8 to differentiate better between the effects due to flow or to calcium antagonism. In the reverse experiments PAF was the first inducer used to stimulate release followed by bradykinin. In these experiments (Table 4) the flow rate was again greatly reduced by the time the second stimulator was perfused. In this case the values obtained for PAF induced t-PA release were not significantly reduced but those for bradykinin induced release were (Table 4). In a previous paper (24) we found that reduction in flow rate was accompanied by a proportional reduction in t-PA release. In Fig. 2 the flow rates, and the amounts of t-PA released, are shown (as percentage of controls) for bradykinin- and PAF-stimulated t-PA release in the presence of calmidazolium, TMB-8, reverse TMB-8 and TFP. As can be seen, for calmidazolium and TMB-8 a good correlation ($r = 0.864$; $n = 7$; $p < 0.01$) existed between mean residual flow rate and mean t-PA release, suggesting that the reduction in t-PA release found was due to the reduction in flow for these compounds. TFP caused some reduction in flow, but a much larger reduction in t-PA release than anticipated on the basis of flow reduction (Fig. 2). Together these data suggest that the effects of calmidazolium and TMB-8 on t-PA release were largely indirect, and did not necessarily reflect an effect of these compounds on t-PA release mechanisms.

The influence of calcium on the release of vWF.

In previous papers (17,24) we have shown that vWF was also released in parallel with t-PA, and that its release was stimulated by the same compounds that stimulate t-PA release and followed a very similar time course to that of t-PA.

The calcium ionophore A-23187 (2 μM) was found to induce vWF release in a similar time course to that of t-PA (see ref. 24).

In the experiments involving the removal of Ca^{++} from the perfusion solution the same effects seen for the PAF-induced t-PA release (Fig. 1a,b) were seen for the PAF-induced vWF release (Fig. 1c,d). Addition of EGTA (1 mM) to the Ca^{++}-free Tyrode/BSA solution again produced a more rapid inhibition of vWF release, as seen for t-PA.

$LaCl_3$ (20 μM and 200 μM), however, had no effect on the vWF release induced by either bradykinin or PAF (Table 5), in contrast to the results seen with t-PA (Table 2).

The calcium channel blockers verapamil and diltiazem had no significant inhibitory effects on the induced release of vWF (Table 5). Diltiazem even showed an increase in the bradykinin-induced vWF release compared to control levels.

The calcium agonist BAY K-8644 (1 μM) reduced (by approximately 50%) the vWF-induced release by both bradykinin and PAF (Table 5).

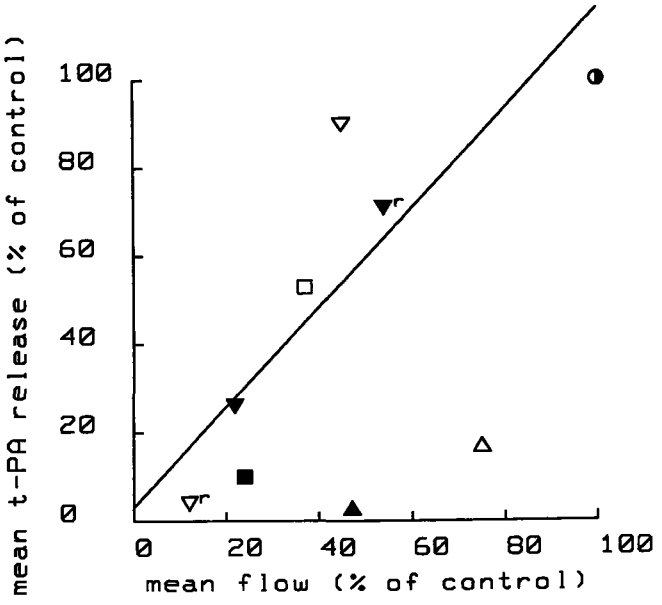

Fig. 2. Scatter plot of mean perfusate flow (abscissa) and mean t-PA release for the control group (o) and for the groups pretreated with calmidazolium (□), TMB-8 (▽), reverse TMB-8 (▽^{r}) or TFP (△). The drawn line represents the least squares regression line for the control, TMB-8 and calmidazolium groups (n = 7; r = 0.864; p < 0.01). Open symbols indicate stimulation with bradykinin, closed symbols indicate stimulation with PAF. Data from Table 4, expressed as percent of the control group.

Table 5. Effect of calcium entry blockers, calmodulin antagonists, and calcium agonist and antagonist on vWF induced release.

Compound	vWF (U/ml) release induced by bradykinin (0.8 μM)	vWF (U/ml) release induced by PAF (5 nM)	Number of experiments
Controls	1.38 ± 0.57*	2.12 ± 0.53	7
$LaCl_3$ (20 μM)	1.65 ± 0.81	1.90 ± 0.15	2
$LaCl_3$ (200 μM)	1.54 ± 0.21	1.91 ± 0.34	4
Verapamil (10 μM)	1.48 ± 0.54	1.53 ± 0.40	4
Diltiazem (20 μM)	2.35 ± 0.27	2.01 ± 0.28	3
BAY K-8644 (1 μM)	0.70 ± 0.09	1.13 ± 0.05**	4
Calmidazolium (10 μM)	1.62 ± 0.12	2.02 ± 0.25	4
TFP (50 μM)	0.55 ± 0.26	0.26 ± 0.22**	3
TMB-8 (70 μM)	0.98 ± 0.35	0.64 ± 0.13**	6

* Mean ± s.d. (peak value of 5 min perfusion periods).

** Significantly different from control (p < 0.01) by one-way ANOVA, followed by Bonferroni's modified t-test.

The calmodulin antagonist TFP (50 μM) showed the same effect on vWF release as on t-PA release, significantly reducing the release of both proteins (Table 5; compare Table 4).

Calmidazolium (10 μM), the other calmodulin antagonist tested, induced its own vWF release, as it did for t-PA. This release was again very similar to that seen for bradykinin-induced release (1.48 ± 0.46 U/ml). The release of vWF was unaffected by the addition of calmidazolium (Table 5), which suggests that flow reduction does not affect vWF release as it does t-PA release (cf Table 4 and Fig. 2).

TMB-8 (10 μM) reduced the induced release of vWF. TMB-8 had a stronger reducing effect on the PAF-induced than on bradykinin-reduced release of vWF, whether PAF was used as the first (Table 5) or the second (not shown) stimulatory compound.

DISCUSSION

In this study we investigated the involvement of calcium and calmodulin in the sequence of events leading to the acute release of t-PA and vWF from the vascular endothelium in the perfused rat hindleg model. In previous papers (8,24) we had suggested that the release of t-PA may be similar to that of EDRF and PGI_2, since all three share a similar array of release-stimulating compounds. A recent review by Adams et al. (1) on difference in the role of Ca^{++} in release of EDRF and PGI_2, suggests that EDRF release is more dependent on the presence of extracellular Ca^{++} and on Ca^{++} influx, while PGI_2 synthesis is more dependent on intracellular Ca^{++} levels (see also 15 and 27). Our data suggested that, similar to the release of EDRF, t-PA release was dependent on Ca^{++} influx. The Ca^{++}-dependency of vWF release is similar, though not identical, to that of t-PA release.

Firstly, the acute release of both t-PA and vWF in our system required the presence of extracellular calcium and was totally abolished in the presence of a Ca^{++}-free, EGTA-containing, perfusion solution. Secondly, the inorganic calcium entry blocker $LaCl_3$ strongly reduced the induced release of t-PA, suggesting that Ca^{++} influx was necessary for this t-PA release. Thirdly, the calcium ionophore A-23187 induced - calcium-dependently - release of t-PA and vWF.

In our *ex vivo* model the acute release of t-PA and vWF from intact endothelial cells thus required extracellular calcium, as does the release of vWF (and protein S) from cultured endothelial cells (4,14,21). In cultured endothelial cells stimuli such as thrombin, histamine, PAF and bradykinin induce similar initial, i.e. during the time period of maximal release of two minutes, increases in intracellular calcium concentrations in the presence and absence of extracellular calcium (11,12,18). As shown by Schilling et al. (19), maximal Ca-influx occurs in bradykinin-stimulated endothelial cells during the first

90 sec of stimulation. Calcium fluxes over the plasma membrane thus seem to be a more important determinant of t-PA and vWF release than the increase in intracellular calcium. De Groot et al. (4) also showed that 3-deazaadenosine, an inhibitor of phospholipid methylation, blocks in cultured endothelial cells both calcium influx and release of vWF.

Increases in phosphoinositides are, in endothelial cells, independent of extracellular calcium (12,13). Also, A-23187 does not increase phosphoinositide concentrations (6,12,13), suggesting that phospholipase C activation (and the resulting release of intracellular calcium by IP_3) is not required for acute t-PA and vWF release to occur. Probably, the activation of protein kinase C by any diacylglycerol formed is also of little importance, a suggestion strengthened by the observation (24) that the protein kinase C activator phorbol myristate acetate induced little release of t-PA and vWF, and did not potentiate PAF-induced release.

TMB-8 significantly reduced the induced release of vWF by PAF though not that induced by bradykinin. TMB-8 did inhibit prostacyclin release in cultured bovine aortic endothelial cells (15) but had no significant effect on bradykinin-induced EDRF release and production (27). As discussed in the results, TMB-8 did not appear to have a direct effect on t-PA release in our system. The information given above suggests that vWF release, like that of prostacyclin, was inhibited by TMB-8, which has no effect on induced EDRF release or induced t-PA release from endothelial cells.

Studies of voltage-sensitive calcium channels have provided evidence for and against their presence in endothelial cells (for review, see 16). The use of the calcium agonist BAY K-8644 and the calcium channel blockers verapamil and diltiazem in our perfusion system did not provide any evidence for the involvement of voltage-operated L-type calcium channels. The compound BAY K-8644 did have an inhibitory effect on the induced release of vWF by both bradykinin and PAF, however, this effect is not one of an agonist, as expected at the concentration used. As this compound did not induce any t-PA or vWF release on its own, or even enhance the induced release of both proteins by bradykinin and/or PAF, one can assume that there are no voltage-operated channels involved in release, or even present, in endothelial cells.

TFP, a calmodulin antagonist, was found to be an effective inhibitor of the acute release of t-PA and vWF, suggesting that calmodulin is involved in the sequence of events leading to the acute release of both t-PA and vWF.

Calmidazolium, another compound which inhibits calmodulin-mediated events, induced the acute release by both t-PA and vWF in substantial amounts on its own. Calmidazolium also induced severe reduction in perfusate flow, obscuring any potential other effect through calmodulin inhibition. The cause of this unexpected finding is not fully understood but may be partially explained by the finding of Busse and Mülsch (2) that calmidazolium did not inhibit nitric oxide synthesis in the cytosol (as other

calmodulin antagonists did) but showed a stimulatory effect on EDRF release from intact cells. They suggest that this effect is caused by the membrane-pertubing property of calmidazolium resulting in an increased Ca^{++} influx into endothelial cells.
Lanthanum chloride (25) was very effective in blocking the induced release of t-PA. However, this was not found to be the case for vWF release, which remained totally unaffected in the presence of lanthanum chloride. Possibly the acute release of vWF in our system requires less calcium influx than the acute release of t-PA, although such a difference does not show in the calcium-depletion experiment of Figs. 1.
Another difference relates to the effect of the calcium antagonists verapamil and diltiazem, which reduce PAF-induced t-PA release, but not vWF-release. A similar observation has been made previously for the endothelin-induced release of t-PA and vWF, in which verapamil, diltiazem and nifedipine also reduced (by 50%) the release of t-PA only (17).

To conclude, one can briefly summarize the data reported in this study as follows. The acute release of t-PA and vWF does require the presence of extracellular calcium and Ca^{++} influx into the endothelial cells. Calcium L-channel blockers were found to have little effect in our system, as did the calcium channel agonist BAY K-8644, supporting the theory that there are no voltage-operated calcium channels in endothelial cells. The release of t-PA and vWF may also be dependent on (TFP-sensitive) calmodulin-dependent pathway though the study of this aspect was hampered by aspecific effects of calmidazolium on release and flow. The precise mechanisms of the acute release of t-PA and vWF from endothelial cells is still not fully understood, but the *in vivo* data provided in this paper support the suggestions based on *in vitro* observations that calcium influx plays an essential role in this acute release mechanism.

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CHAPTER 7

MODULATION OF THE ACUTE RELEASE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND VON WILLEBRAND FACTOR FROM ENDOTHELIAL CELLS BY CYCLIC NUCLEOTIDES

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ABSTRACT

The modulation of the induced acute release of tissue-type plasminogen activator (t-PA) and of von Willebrand factor (vWF) by compounds affecting cyclic nucleotide levels was studied. The experimental system used was the isolated rat hindleg region perfused with Tyrode's. In this system, a rapid (within 1-2 min) transient release of t-PA and vWF was induced by adding either platelet-activating factor (PAF; 5 nM) or bradykinin (BRA; 0.8 μM) to the Tyrode's.

The guanylate cyclase activators sodiumnitroprusside and atrial natriuretic factor reduced the induced release of both t-PA and vWF. The adenylate cyclase activator forskolin had no effect on BRA-induced release. However, forskolin reduced the PAF-induced release of t-PA and potentiated the simultaneously-induced vWF release. Isoproterenol enhanced BRA-induced release without affecting PAF-induced release.

The following phosphodiesterase inhibitors were perfused either alone, or in combination with a cyclic nucleotide activator: SKF 94120 and Rolipram (c-AMP selective), M & B 22948 (c-GMP selective), and isobutylmethylxanthine (non-selective). None of these compounds induced any t-PA or vWF release on their own. Isobutylmethylxanthine and M & B 22948 did not affect induced release. Rolipram and SKF 94120 had no effect at the lower dosage (10 μM), but tended to reduce the induced release of t-PA and vWF at higher dosages (100 and 200 μM).

Our results suggest that cyclic nucleotides did not, by themselves, induce the acute endothelial release of t-PA and vWF in perfused hindlegs, but that altering their levels modulated this release.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) and von Willebrand factor (vWF) are two proteins which are concomitantly released from the vascular endothelium. In our model of the perfused rat hindleg t-PA and vWF release from endothelial cells can be induced by a variety of stimuli including bradykinin, thrombin, histamine, A-23187, platelet-activating factor (PAF), leukotrienes and endothelin (6,8,20,27). Protein synthesis is not required for the acute release of t-PA and vWF; however, the presence of extracellular calcium is essential (28,29).

Regulation of cellular responses by cyclic nucleotides has been the subject of intense investigation over the past thirty years. The cyclic nucleotides cAMP and, more recently, cGMP, have been implicated as second messengers mediating hormonal effects on a number of biological processes, including secretion. As the above-mentioned compounds that induce the acute release of t-PA and vWF are known to affect cyclic nucleotide levels in endothelial cells (see e.g. 2,3,18,22,24), cyclic nucleotides might also be involved in the acutely-induced release from endothelial cells.

The intracellular concentrations of the cyclic nucleotides appear to be regulated by two classes of enzymes, the cyclase enzymes and the cyclic nucleotide phosphodiesterases (PDE). Forskolin (FSK), a diterpene, directly stimulates adenylate cyclase and has been extensively used to elevate cAMP levels (15,25). The β-adrenoceptor agonist isoproterenol (ISO) also activates adenylate cyclase in many tissues including endothelial cells (4,14,22). The conversion of guanosine triphosphate to cGMP can be catalysed by at least two forms of guanylate cyclase, a soluble form activated by e.g. nitrovasodilators, and a particulate form activated by atrial natriuretic factor (ANF). Both soluble and particulate guanylate cyclases are present in endothelial cells (see e.g. 16,18,23).

Multiple forms of PDEs have been isolated, whose distribution is tissue specific. The use of selective inhibitors has helped to characterize and isolate these different PDE forms (1). Little information is available about the PDEs present in endothelial cells; an analysis of PDEs present in cultured pig aortic endothelial cells was only recently published (26). In this study we have used several PDE inhibitors together with the cyclase activators mentioned above: 3-isobutyl-1-methylxanthine (IBMX), a non-selective PDE inhibitor; M & B 22948*, a cGMP specific inhibitor; and SKF 94120 and Rolipram, which are cAMP specific.

In view of the observations mentiond above, we decided to investigate the possible involvement of the cyclic nucleotide pathways in the acute release of both t-PA and vWF in the perfused rat hindleg model.

*Note: Trade name Zaprinast.

METHODS

Male Wistar rats (Broekman Institute, Helmond, The Netherlands), weighing 250-300 g, anaesthetized using pentobarbital (Nembutal, 60 mg/kg intraperitoneally), were used in the following experiments.

Rat hindleg perfusion.

The perfused rat hindleg system was used to study the acute release of t-PA and vWF from a perfused vascular bed, as described in detail elsewhere (27,28). Briefly: the rat hindleg region was perfused through the aorta with oxygenated Tyrodes' salt solution containing 0.1 mg/ml bovine serum albumin (Tyrode/BSA), pH 7.5, at 37°C using a roller-pump at a constant flow of 9-10 ml/min. Perfusate samples were collected from an out-flow cannula inserted into the vena cava. A 30 min perfusion period using Tyrode/BSA was allowed to clear residual blood from the blood vessel system in control experiments. Two compounds were then used to stimulate release in each experimental animal; first bradykinin (0.8 μM) was perfused through the system for 5 min, followed by a 5 min wash-out period before PAF (5 nM) was perfused for 5 min. In experiments involving PDE inhibitors, the drug under investigation was added to the Tyrode/BSA solution after 10 min and perfused for 20 min before, and during, the periods of stimulating release. In experiments involving the cyclase activators, the drug under investigation was added to the Tyrode/BSA solution after 25 min and perfused for 5 min before, and during, the periods of stimulation. Sample collections were taken every minute for 30 sec, kept on ice until the experiment was completed, and then centrifuged (3000 g for 10 min). The supernatant was collected and mixed 1:9 with a solution containing 0.5 M Tris/HCl (pH 7.5) and 1% Triton X-100, and either assayed immediately or stored at -20°C.

For the drugs used in a specific solvent (e.g. ethanol), solvent control experiments were conducted. However, none of the solvent controls were significantly different from control experiments, so the data was pooled together and is presented as "control" experiments.

Spectrophotometric plasminogen activator (PA) assay.

The PA activity of the perfusate samples was determined by the indirect spectrophotometric rate assay described by Verheijen et al. (30). The nature of the PA activity was determined to be t-PA as described previously (8,27). Dilutions of human melanoma t-PA were run in each plate for calibration, and the activity of the samples will expressed in Units, one Unit (of rat t-PA) being equivalent in our assay to one international unit of human t-PA (International Standard batch 83/517).

ELISA determination for von Willebrand Factor (vWF).

The concentration of vWF in the perfusate samples was determined using an ELISA assay adapted from Ingerslev (13) as detailed elsewhere (29). Briefly, 96-well microtiter plates were coated with rabbit anti-human vWF Ig and left overnight at 4°C. After rinsing, the perfusate samples (25 μl) and 74 μl of PBS-Tween buffer (pH 7.2) were added. Bound antigen was detected using peroxidase-conjugated rabbit anti-human vWF Ig. Absorbances were measured at 450 nm in a Titertek multiscan spectrophotometer. Rat pooled plasma in a range of 0.05 to 5.0% (v/v) was used in each plate for calibration. The concentration of vWF will be expressed as Units, one hundred Units being equivalent to the amount of vWF present in one ml of pooled rat plasma.

MATERIALS

Platelet-activating factor (PAF) was obtained from Bachem (Bubendorf, Switzerland). Nembutal was from Sanofi (Paris, France). Bradykinin, N-nitro-L-arginine methyl ester, bovine serum albumin, forskolin (FSK), rat atriopeptin II (ANF), 8-bromoguanosine 3'5'-cyclic monophosphate (8-br-cGMP), 8-bromoadenosine 3'5'-cyclic monophosphate (8-br-cAMP) were from Sigma (St. Louis, U.S.A.). Sodium nitroprusside (SNP) was from Merck (Darmstadt, Germany). Isoproterenol (ISO) was from Serva (Heidelberg, Germany). 3-Isobutyl-1-methylxanthine (IBMX) was from Janssen Chimica (Beerse, Belgium). Rabbit anti-human vWF immunoglobulin (Ig) and peroxidase-conjugated rabbit anti-human vWF Ig came from Dakopatts (Copenhagen, Denmark). Purified human t-PA (two-chain) was a gift from Dr. J.H. Verheijen of the Gaubius Institute. Rolipram (4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone) was a gift from Schering (Berlin, F.R.G.), M & B 22948 (2-0-propoxyphenyl-8-azapurin-6-one) was a gift from May and Baker Ltd. (Dagenham, U.K.), and SKF 94120 (5-(4-acetamidophenyl)pyrazin-2(1H)-one) was a gift from Smith, Kline and French Ltd. (Welwyn Gardin City, U.K.).

The materials used in the spectrophotometric assay have been detailed previously (30). All other materials were of analytical grade.

STATISTICS

Results were analysed by one-way ANOVA, followed by Bonferroni's modified t-test, or by Student's t-test, as indicated below. Differences were considered significant if p (two-sided) was < 0.05.

RESULTS

The effects of guanylate cyclase activators on t-PA and vWF release from perfused rat hindlegs.

Bradykinin and PAF are used routinely to sequentially induce acute release of t-PA and vWF in our system. This release is rapid (peaking within 1-2 min) and transient (see e.g. 28,29), the release of both proteins following the same time-course. Perfusion using only Tyrode/BSA solution does not result in detectable baseline release of either protein.

Perfusion with SNP (1 mM) or with ANF (20 nM) did not induce any t-PA or vWF release in our model. However, both drugs reduced the induced release of t-PA and vWF (Table 1), the effect of SNP being stronger than that of ANF.

Table 1. Effects of guanylate cyclase activators on the induced release of t-PA and vWF.

t-PA release (U/ml) (peak values of a 5 min perfusion period)			
Compounds	Release induced by bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.66 ± 0.38*	2.34 ± 0.40	30
SNP (1 mM)	1.14 ± 0.40^{+}	0.64 ± 0.29^{+}	4
ANF (20 nM)	1.12 ± 0.20^{+}	1.82 ± 0.27^{+}	4
vWF release (U/ml) (peak values of a 5 min perfusion period)			
Compounds	Release induced by bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.00 ± 0.23	1.16 ± 0.35	30
SNP (1 mM)	0.16 ± 0.10^{+}	0.13 ± 0.05^{+}	4
ANF (20 nM)	0.60 ± 0.16^{+}	0.77 ± 0.12^{+}	4

* Mean ± s.d.

$^{+}$ Significantly different from control values by one-way ANOVA, followed by Bonferroni's modified t-test.

Nitro-arginine (an inhibitor of NO formation and thus of NO-induced cGMP synthesis, ref. 19) at 50 μM or 100 μM did not induce any t-PA or vWF release on its own and had also no effect on the induced release of t-PA and vWF by either bradykinin or PAF (data not shown).

The effects of adenylate cyclase activators on t-PA and vWF release from perfused rat hindlegs.

FSK (10 μM) and ISO (10 μM) did not release any t-PA or vWF themselves. At a concentration of 40 μM, ISO had previously been found to be a weak, inconsistent inducer of t-PA release. (see ref. 6).

Table 2. Effects of adenylate cyclase activators on the induced release of t-PA and vWF.

t-PA release (U/ml) (peak values of a 5 min perfusion period)

Compounds	Release induced by bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.66 ± 0.38*	2.34 ± 0.40	30
Forskolin (10 μM)	1.60 ± 0.25	1.62 ± 0.40+	10
Isoproterenol (10 μM)	2.88 ± 0.33+	2.26 ± 0.70	4

vWF release (U/ml) (peak values of a 5 min perfusion period)

Compounds	Release induced by bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.00 ± 0.23	1.16 ± 0.35	30
Forskolin (10 μM)	1.02 ± 0.25	2.19 ± 0.61+	10
Isoproterenol (10 μM)	1.37 ± 0.44+	0.96 ± 0.15	4

* Mean ± s.d.

\+ Significantly different from control values by one-way ANOVA, followed by Bonferroni's modified t-test.

ISO had no effect on the release of t-PA and vWF induced by PAF. ISO did, however, significantly increase the release of t-PA and vWF induced by bradykinin (Table 2). FSK did not affect the BRA-induced release of either t-PA or vWF. The effects of FSK on the PAF-induced release of the two proteins were divergent: FSK increased the PAF-induced release of vWF and simultaneously reduced the PAF-induced release of t-PA (Table 2). The effects were seen both in the peak values collected in a five minute perfusion (Table 2) and in the total sum amount of t-PA (or vWF) released over a five minute perfusion period (calculated as the sum of the concentrations in the five samples obtained). The effects on peak levels of release were thus not due to a change in the time-course of release.

The effect of cyclic nucleotide analogues on the release of t-PA and vWF.

The cell permeant analogue of cAMP, 8-br-cAMP, was perfused for 15 min at a dose of 1 mM; no release of either t-PA or vWF was observed. 8-Br-cGMP, the lipid soluble analogue of cGMP, was also perfused through the rat hindleg region for 15 min (at 1 mM); this too induced no release of either t-PA of vWF. 8-Br-cAMP had no significant effect on t-PA release induced by either BRA or PAF (Table 3). However, it did increase the release of vWF by BRA and PAF. This increase in the induced release of vWF was thus similar to the effect of FSK.

In contrast, 8-br-cGMP had no effect on the induced release of vWF by either BRA or PAF. However, it did significantly increase the t-PA induced release by PAF, though not that by BRA (Table 3).

Effects of PDE inhibitors on the release of t-PA and vWF.

The PDE inhibitors M & B 22948, IBMX, SFK 94120 and Rolipram, were investigated at doses of 10 μM, 100 μM and 200 μM. None of these inhibitors released any t-PA or vWF from the rat hindleg. The modulation of the induced release of t-PA and vWF by these inhibitors was investigated next. Figure 1 summarizes the results found in this series of experiments. This figure only shows the induced release of t-PA, as the data obtained for the vWF induced release followed the same trend as that obtained for t-PA. IBMX, a non-selective PDE inhibitor, and M & B 22948, a cGMP-selective PDE inhibitor, did not significantly affect the induced release by either BRA or PAF at 10 μM, 100 μM and 200 μM concentrations (Fig. 1).

Table 3. Effects of cyclic nucleotide analogues on the induced release of t-PA and vWF.

t-PA release (U/ml) (peak values of a 5 min perfusion period)			
Compounds	Release induced by bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.66 ± 0.38*	2.34 ± 0.40	30
8-Br-cAMP (1 mM)	2.00 ± 0.31	2.52 ± 0.23	4
8-Br-cAMP (1 mM)	1.84 ± 0.30	3.21 ± 0.11+	4

vWF release (U/ml) (peak values of a 5 min perfusion period)			
Compounds	Release induced by bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.00 ± 0.23	1.16 ± 0.35	30
8-Br-cAMP (1 mM)	1.72 ± 0.54+	2.27 ± 0.11+	4
8-Br-cAMP (1 mM)	1.15 ± 0.46	1.44 ± 0.38	4

* Mean ± s.d.

\+ Significantly different from control values by one-way ANOVA, followed by Bonferroni's modified t-test.

The effects of the cAMP-selective PDE inhibitors Rolipram and SKF 94120 were more complex. At 10 μM, no significant effect was seen (Fig. 1). For BRA-induced release, both inhibitors significantly diminished release at 100 μM, an effect that was largely reversed at 200 μM. For PAF-induced release, similar effects as for BRA were seen with SKF 94120. Rolipram, however, induced a highly significant, progressive reduction of both t-PA release and vWF release induced by PAF (Fig. 1). The effect of 200 μM Rolipram was also tested in experiments where the sequence of activators was reversed, i.e. PAF was used as the first stimulus, followed by BRA. The same results were obtained, PAF-induced release of t-PA and VWF was strongly reduced while BRA-induced release remained unaffected (data not shown).

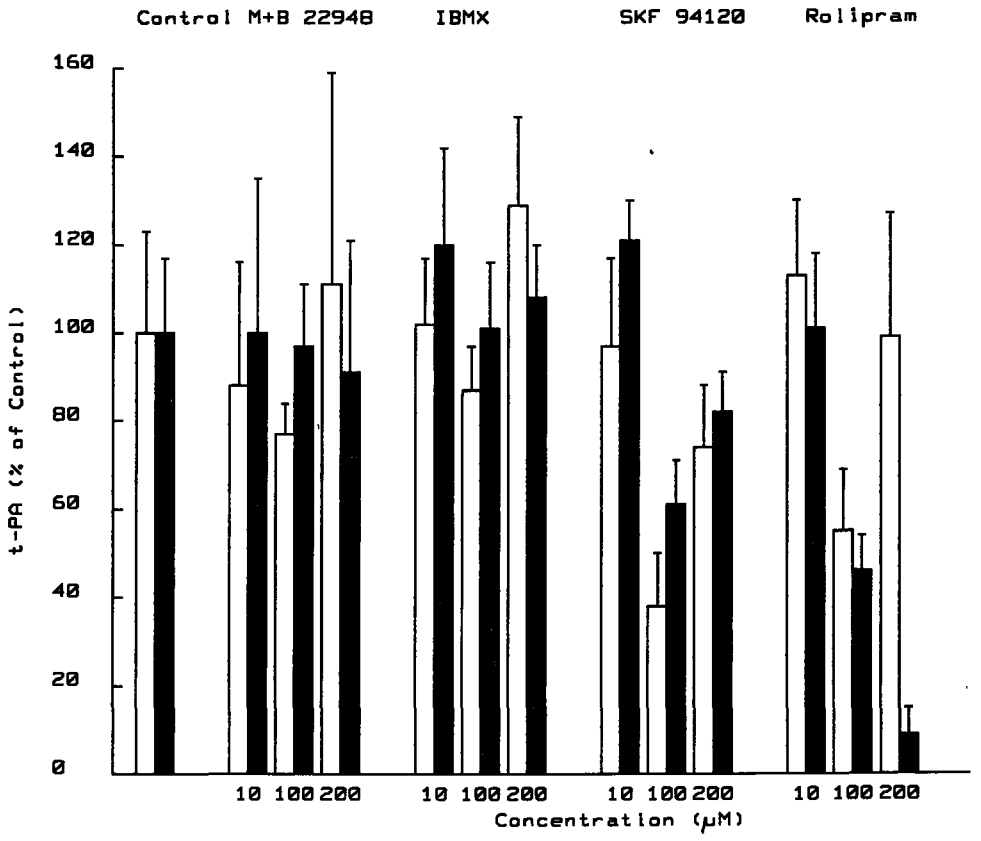

Fig. 1. The effects of PDE inhibitors on the release of t-PA. Bar graphs show the release of t-PA, as percentage of control values, by bradykinin (0.8 μM, □), followed by - after a 5 min wash-out - PAF (5 nM, ■). Data shown are means ± s.d. for 30 control perfusions and for a minimum of 4 perfusions performed in the presence of the PDE inhibitor at concentrations of 10 μM, 100 μM and 200 μM.

The effects of combination experiments on the release of t-PA and vWF.

Experiments were conducted that involved the combination of a PDE inhibitor and a cyclase activator. Four different combinations were run: M & B 22948 (100 μM) and SNP (1 mM), IBMX (100 μM) and FSK (10 μM), SKF 94120 (100 μM) and FSK (10 μM) and Rolipram (100 μM) and FSK (10 μM). The data from these experiments are shown in Figure 2. None of these combinations produced any t-PA or vWF release on their own. The combination of M & B 22948 and SNP on the induced release of t-PA and vWF, showed the same results as those observed by SNP alone (Fig. 2 and Table 1).

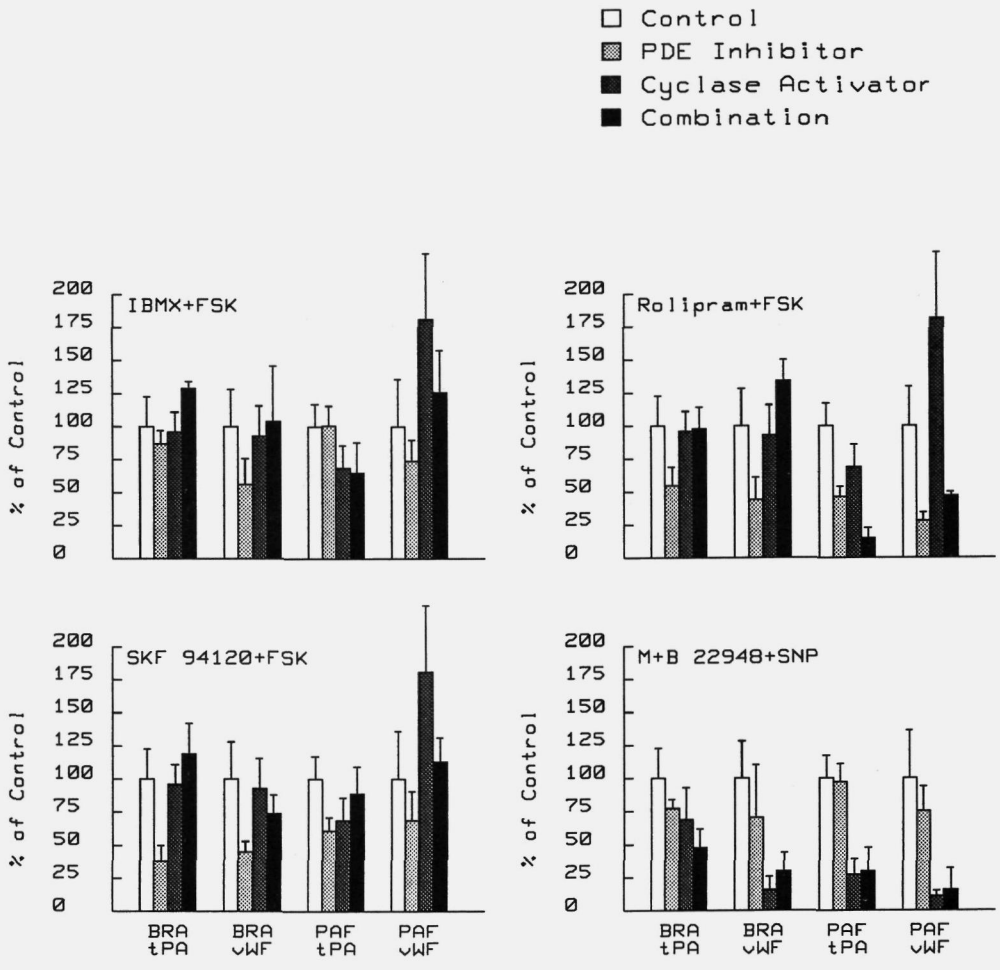

Fig. 2. The effect of combination of a cyclase activator with a PDE inhibitor on release.
Bar graphs show the release of t-PA and vWF induced by bradykinin (BRA, 0.8 μM) and PAF (5 nM) for experiments combining a PDE inhibitor (100 μM) and a cyclase activator (FSK, 10 μM; or SNP, 1 mM). The effect of each compound on the induced release of t-PA and vWF by bradykinin and PAF is shown as percentage of control values separately for each compound and for the combination. Data shown are means $\pm$ s.d. for 30 control perfusions and for a minimum of 4 perfusions for each experimental compound.

The BRA- and PAF-induced release of both t-PA and vWF were significantly reduced. The combination of IBMX and FSK showed the same results as those seen with FSK alone. There was no effect on the BRA-induced release of both t-PA and vWF, while the PAF-induced t-PA release was significantly reduced and the simultaneous vWF release was elevated (Fig. 2 and Table 2). In the experiments combining SFK 94120 and FSK, the combination results obtained were close to the control values. No significant increases or decreases were seen in the BRA- and PAF-induced release of either t-PA or vWF. In the experiments combining Rolipram and FSK, the reducing effects on BRA-induced release by Rolipram (100 μM) alone were removed (Fig. 2), vWF being even slightly increased. The effects of the combination Rolipram-FSK on PAF-induced release closely mimicked those obtained using Rolipram alone. Both t-PA and vWF release were strongly reduced (Fig. 2), similar to the inhibition obtained with Rolipram alone at 200 μM (Figs 1 and 2). In an experiment using a reversed order of stimuli, the same effects were seen as in the normal experiments (data not shown).

DISCUSSION

Neither cyclase activators such as FSK, ISO, SNP and ANF, nor the cyclic nucleotide analogs 8-br-cAMP and 8-br-cGMP, nor PDE inhibbitors such as IBMX and Rolipram, nor combinations of these compounds induced, in the perfused rat hindleg, the release of either t-PA or vWF. It is thus likely that changes in cyclic nucleotide levels in themselves are not sufficient to induce the acute release from endothelial cells of t-PA or vWF. These data would agree with the clinical observation (17) that cAMP infusion does not influence plasma fibrinolytic activity in humans. In contrast, once triggered by compounds such as BRA or PAF, the induced release of t-PA and vWF was inhibited by SNP and ANF, two activators of endothelial guanylate cyclase. This would suggest that ongoing endothelial release is down-regulated by increased cGMP levels. A similar inhibitory effect on the release of endothelium-dependent relaxing factor (EDRF) from endothelial cells has been described for ANF and 8-br-cGMP (9,11), while nitric oxide, another activator of guanylate cyclase, reduces endothelial prostacyclin production (5). In a previous paper (7) we suggested that the induction of the release of EDRF and of t-PA, and that of prostacyclin production share, in endothelial cells, a common metabolic pathway. The similar inhibitory effects of the cGMP-inducing agents mentioned above would agree with this suggestion.

In contrast to these effects, exogenously applied 8-br-cGMP had no inhibitory effect, but even slightly enhanced release (Table 3). No obvious explanation for this discordant result is available. Possibly, cGMP has to be compartmentalized to have an inhibitory effect in endothelial cells, or 8-br-cGMP is handled in a different way than native cGMP

(10). The cGMP-selective PDE inhibitor M & B 22948 also had no significant effect on release, and did not enhance the effect of SNP. These results were likely due to the absence in rat endothelial cells of a M & B 22948-sensitive PDE, as has recently been described for porcine endothelial cells (26). As compounds that induce release also in general induce EDRF (7), which in turn will induce cGMP in endothelial cells (18) the rapid, physiological, down-regulation of release from two min after the initiation of release onwards might have been due to cGMP. However, nitro-arginine, an inhibitor of EDRF formation (19), did not affect either the peak value, or the time course of the release of t-PA or vWF.

No clear-cut picture of the putative role of cAMP in endothelial release could be obtained from the present experiments. FSK influenced only PAF-induced release, while ISO (at 10 μM) affected only BRA-induced release, and 8-br-cAMP increased vWF release only (Tables 2 and 3). In interpreting the effect of ISO it should be remembered that - at the higher concentration of 40 μM - ISO itself is a weak, inconsistent inducer of release (6), so that its effect may have been due to an effect as an inducer per se, and not as a modulating compound, thus possibly only affecting the weaker inducer BRA but not the stronger inducer PAF. FSK, though widely used as a direct activator of adenylate cyclase (25), is known to have several side-effects not related to its activation of adenylate cyclase (15). These side-effects include effects on ion channels (12), while we have shown previously that calcium fluxes are essential for release to occur (20, and unpublished data). The most clear-cut effect observed was that of Rolipram, which differentiated between the mode of action of BRA and that of PAF. Rolipram reduced the PAF-induced release of both t-PA and vWF dose-dependently (Figs. 1 and 2); however, BRA-induced release was affected inconsistently. As Rolipram has no known PAF-antagonistic properties, and as FSK enhanced the effect of Rolipram, resulting in an effect of Rolipram (100 μM) and FSK (10 μM) combined that was identical to the effect of 200 μM Rolipram alone (Fig. 2), it is likely that the said effect on PAF-induced release is due to an effect via cAMP. This would suggest that cAMP is more important in the mediation of PAF-induced release than in that of BRA. However, as was the case for 8-br-cGMP, this interpretation leaves the effect of 8-br-cAMP unexplained. In summary, our data suggest that the cyclic nucleotides cAMP and cGMP by themselves do not induce acute release of t-PA or vWF from endothelial cells. Cyclic GMP, however, down-regulates the acute release of t-PA and vWF, while the role of cAMP, if any, is still undetermined and may be restricted to PAF-induced release only.

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CHAPTER 8

THE EFFECT OF PENTOXIFYLLINE (TRENTAL) AND TWO ANALOGS, BL 194 AND HWA 448, ON THE RELEASE OF PLASMINOGEN ACTIVATORS AND VON WILLEBRAND FACTOR IN RATS

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SUMMARY

The effect on fibrinolytic components of pentoxifylline (Trental), of its first metabolite BL 194 (penthydroxyfylline) and of its analog HWA 448 (torbafylline) were studied in rats.

BL 194, though not pentoxifylline and HWA 448, significantly enhanced the induced release by platelet-activating factor (PAF) of tissue-type plasminogen activator (t-PA) from isolated perfused rat hindlegs. In contrast, the simultaneously induced release of von Willebrand factor (vWF) was reduced by BL 194. The effect of BL 194 on PAF-induced release of t-PA and vWF could be mimicked by isobutylmethylxanthine (IBMX), an inhibitor of phosphodiesterases.

In vivo, BL 194 and pentoxifylline did not affect baseline levels of plasma t-PA and PA inhibitor activity, nor did these compounds affect the *in vivo* induction of t-PA release by PAF. Similarly, the induction by endotoxin of PA inhibitor activity was not influenced by pentoxifylline or BL 194.

By its opposite effects on t-PA and vWF release, BL 194 might favourably influence the thrombotic balance.

INTRODUCTION

Pentoxifylline (trade name "Trental"; 3,7,dimethyl-1-(5-oxohexyl)xanthine) is used clinically to improve peripheral vascular circulation and to relieve intermittent claudication and increase walking distance (1). The mechanism of action of the drug is

believed to mainly involve the prevention of platelet aggregation and reduction of blood viscosity possibly by inducing changes in flexibility and aggregation of erythrocytes (2).

Pentoxifylline has been found to enhance both intracellular levels of cyclic adenosine monophosphate (cAMP), presumably by phosphodiesterase (PDE) inhibition, and also cAMP-dependent protein kinase activity (3,4). Further studies using pentoxifylline have shown that pentoxifylline stimulates vascular prostacyclin (PGI2) formation in animals and in human umbilical arteries and veins *in vitro* (5,6), and also in humans *in vivo* (7).

Recently, pentoxifylline was found to enhance polymorphonuclear leukocyte (PMN) motility by a direct effect on PMNs (8). Pentoxifylline was also found to attenuate acute lung injury in septic guinea pigs (9), this and the inhibitory effect on neutrophils indicates that pentoxifylline might have a use in the treatment of sepsis and Adult Respiratory Distress Syndrome (10).
Fibrinolytic activity in humans has been shown to increase with repeated administration of pentoxifylline (11,12). Klöcking et al. (13), using both pentoxifylline and its first metabolite BL 194, showed a dose-dependent increase in the release of plasminogen activators from perfused isolated pig ears by BL 194 only. In view of these observations, a detailed study was planned to investigate the effect of pentoxifylline, BL 194, its first metabolite, and HWA 448, one of its analogues, on plasminogen activators, von Willebrand factor (vWF) and plasminogen activator inhibitor-1 (PAI-1) in rats. The acute release of both tissue-type plasminogen activator (t-PA) and vWF was studied using the rat hindleg perfusion model. Experiments were done *in vivo* to ascertain the effects of pentoxifylline and BL 194 on both the induction of PAI activity, and on the plasma levels of t-PA and urokinase-type plasminogen activator (u-PA) in the whole animal.

METHODS

Male Wistar rats (Broekman Institute, Helmond, The Netherlands), weighing 250-350 g, anaesthetized using pentobarbital (Nembutal^{R}, 60 mg/kg intraperitoneally), were used in the following experiments.

Rat hindleg perfusion.
The perfused rat hindleg system was used to study the acute release of t-PA and vWF from a perfused vascular bed, as described in detail elsewhere (14,15). Briefly: the rat hindleg region was perfused through the aorta with oxygenated Tyrode's salt solution containing 0.1 mg/ml bovine serum albumin (BSA), pH 7.5, at 37 °C using a roller-pump at a constant flow of 9-10 ml/min. Perfusate samples were collected from an out-flow

cannula inserted into the vena cava. A 30 min perfusion period using Tyrode/BSA solution was allowed to clear residual blood from the blood vessel system in control experiments. In experiments involving pentoxifylline or analogs, the drug under investigation was added to the Tyrode/BSA solution after 10 mins and perfused for 20 mins before, and during, the period of stimulating release. Two compounds were used to stimulate release in each experimental animal; first bradykinin was perfused through the system for 5 min, followed by a 5 min wash-out period before PAF (platelet-activating factor) was perfused for 5 mins. Sample collections were taken every minute for 30 sec, kept on ice until the experiment was completed, and then centrifuged (3000 g for 10 min). The supernatant was collected and mixed 1:9 with a solution containing 0.5 M Tris/HCl (pH 7.5) and 1% Triton X-100, and either assayed immediately or stored at -20°C.

***In vivo* experiment.**

The doses of pentoxifylline and BL 194 used in the following experiments were based on data and results presented by Weichert and Breddin (16) and Luke and Rocci (17).

1. *The induction of PAI activity by endotoxin* (18)

Rats were anaesthetized with Nembutal and injected i.v. with either pentoxifylline (20 mg/kg), BL 194 (20 mg/kg) or saline (2 ml/kg). One hour later the rats were injected i.v. with endotoxin (10 μg/kg). Three hours after the endotoxin injection, the rats were reanaesthetized and bled by aortic puncture. Plasma samples were prepared by taking nine parts of blood to one part of sodium citrate (3.8% w/v), centrifuged at 4°C for 10 minutes at 2000 x g and stored as platelet-poor plasma at -20°C.

2. *Effect of sub-acute treatment on base-line levels of plasminogen activators*

Rats were injected, i.p., daily for four days with pentoxifylline (50 mg/kg), BL 194 (50 mg/kg) or saline (2.5 ml/kg). On the fourth day the rats were fasted overnight, and on the fifth day, they were bled by aortic puncture and plasma was prepared.

3. *Effect of acute treatment on plasminogen activator release in vivo*

Anaesthetized rats were initially injected (i.v.) with pentoxifylline (50 mg/kg), BL 194 (50 mg/kg) or saline (2.5 ml/kg), and one hour later were injected with PAF (0.4 μg/kg, i.v.). The animals were bled from the aorta exactly one minute after the injection of PAF. Platelet-poor plasma was prepared, and plasma PA activities determined in the euglobulin fraction.

4. *Effect of acute treatment on dilute blood clot lysis times*

Rats were injected i.v. with pentoxifylline (20 mg/kg), BL 194 (20 mg/kg) or saline (2 ml/kg) one hour before PAF (0.5 μg/kg) was injected i.v.. Blood samples were obtained from a cannula in the carotid artery before and at 1, 2, 3, 5, 7 and 10 minutes after injection of PAF.

Blood (0.2 ml) was diluted to 10% in 1.7 ml of 0.12 mol/l sodium acetate (pH 7.4),

clotted with 0.1 ml thrombin (20 U/ml) and incubated at 37°C. Lysis times were read in minutes.

Spectrophotometric plasminogen activator assay.
The PA activity of the perfusate samples was determined by the indirect spectrophotometric rate assay described by Verheijen et al. (19). Dilutions of human melanoma t-PA were run in each plate for calibration, and the activity of the samples will be expressed in Units, one Unit (of rat t-PA) being equivalent in our assay to one international unit (IU) of human t-PA (International Standard batch 83/517).

Spectrophotometric plasminogen activator inhibitor (PAI) assay.
PAI activity was determined in plasma samples by titration with human t-PA followed by spectrophotometric determination of residual t-PA activity as described by Verheijen et al. (20). One Unit of PAI activity represents inhibition of one IU of human t-PA.

Spectrophotometric plasma plasminogen activator activity assay.
Plasma PA activities were determined spectrophotometrically (19) in the plasma euglobulin fraction (pH 6.0, dilution 1:20). The t-PA activity represents the activity quenched by excess anti-rat t-PA IgG, the u-PA activity represents the activity quenched by 100 μM amiloride (21). Results will be expressed in Units, as defined above.

ELISA determination for von Willebrand Factor (vWF).
The presence of vWF in the perfusion samples was determined using an ELISA assay adapted from Ingerslev (22) as detailed elsewhere (23). Briefly, 96-well microtiter plates were coated with rabbit anti-human vWF immunoglobulin and left overnight at 4°C. After rinsing the perfusate samples, 25 μl, and 75 μl of PBS-Tween buffer (pH 7.2) were added. Bound antigen was detected using peroxidase-conjugated rabbit immunoglobulins to human vWF. Absorbances were measured at 450 nm in a Titertek multiscan spectrophotometer. Rat pooled plasma in a range of 0.05 to 5.0% was used in each plate for calibration. The concentration of vWF will be expressed as Units, a hundred Units being equivalent to the amount of vWF present in one ml of pooled rat plasma. The detection limit of the assay was 0.1 U/ml.

MATERIALS

Platelet-activating factor was obtained from Bachem (Bubendorf, Switzerland). Nembutal was from Sanofi (Paris, France). Bovine thrombin from Leo Pharmaceuticals (Ballerup, Denmark). Bradykinin, bovine serum albumin, endotoxin (lipopolysaccharide from E.coli

0128: B12) and amiloride were from Sigma (St. Louis, U.S.A.). Purified human t-PA (two-chain) was a gift from Dr. J.H. Verheijen of the Gaubius Institute. Anti-rat t-PA IgG had been raised against t-PA purified from L2 cells. Rabbit anti-human vWF immunoglobulin and peroxidase-conjugated rabbit immunoglobulins to human vWF came from Dakopatts, Denmark.

3-Isobutyl-1-methylxanthine (IBMX) was from Janssen Chimica, Beerse, Belgium. Pentoxifylline (Trental, 3,7-dimethyl-1-(5-oxohexyl)xanthine), HWA 448 (torbafylline, 7 ethoxymethyl-1-(5-hydroxy-5-methylhexyl)-3-methyl-xanthine), and BL 194 (3,7,dimethyl-1-(5-hydroxyhexyl)-xanthine) were all gifts from Hoechst AG, Wiesbaden, F.R.G.

The materials used in the spectrophotometric PA assay have been detailed elsewhere (19). All other materials were of analytical grade. All compounds to be injected were dissolved in sterile, pyrogen-free saline.

Titertek spectrophotometer and microtiter plates came from Flow Laboratories, Irvine, U.K.

RESULTS

The effects of pentoxifylline, BL 194 and HWA 448 on t-PA and vWF release from perfused rat hindlegs.

Perfusion using only Tyrode/BSA solution did not result in any detectable baseline t-PA or vWF release in our system. Two compounds, such as bradykinin and PAF are used routinely to induce release in our system (24).

Pentoxifylline, BL 194 and HWA 448 did not induce any t-PA or vWF release in our model system when perfused for 20 minutes at a concentration of 10 μM, 50 μM and 100 μM.

Pentoxifylline did not potentiate t-PA release induced by bradykinin (0.8 μM) or PAF (5 nM), as can be seen in Table 1a. However, BL 194 did potentiate the t-PA release by PAF. BL 194 increased both the maximal release of t-PA (Table 1a; Fig. 1) and the total amount of t-PA released over 5 minutes by PAF (calculated as the sum of the concentrations in the 5 samples obtained (Table 1b, Fig. 1). Although BL 194 did not significantly increase the maximal release by bradykinin, its effects reached significance concerning the total amount of t-PA release by bradykinin over 5 min (Table 1b). Increasing the concentration of BL 194 from 10 μM to 50 μM did not show any greater or different effect on the t-PA release seen (data not shown).

Table 1. Effect of pentoxifylline, BL 194 and HWA 448 on the induced release of t-PA from rat hindlegs.

a) t-PA release (U/ml) (peak values of a 5 min perfusion).

Compound	Release induced by Bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.71 ± 0.32*	1.96 ± 0.34	5
Pentoxifylline (10 μM)	1.29 ± 0.17	1.94 ± 0.19	3
BL 194 (10 μM)	2.09 ± 0.46	3.55 ± 0.64**	3
HWA 448 (10 μM)	1.35 ± 0.15	2.28 ± 0.49	3

* Mean ± s.d.
** Significantly different to controls by ANOVA ($p < 0.001$).

b) t-PA release (U/ml) (sum values over the 5 samples of one perfusion).

Compound	Release induced by Bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	3.64 ± 0.61*	4.81 ± 1.18	5
Pentoxifylline (10 μM)	3.56 ± 0.80	4.32 ± 0.61	3
BL 194 (10 μM)	5.71 ± 1.41**	7.24 ± 1.39***	3
HWA 448 (10 μM)	3.77 ± 0.38	5.13 ± 1.06	3

*Mean ± s.d.
**Significantly different to controls by ANOVA ($p < 0.025$).
***Significantly different to controls by ANOVA ($p < 0.05$).

Pentoxifylline had no effect on the vWF release induced by either bradykinin or PAF in the perfused hindleg (Table 2). However, BL 194 was found to affect the release of vWF induced by PAF. In contrast to the t-PA release, which BL 194 potentiated, BL 194 significantly decreased the vWF release induced by PAF (Table 2, Fig. 2).

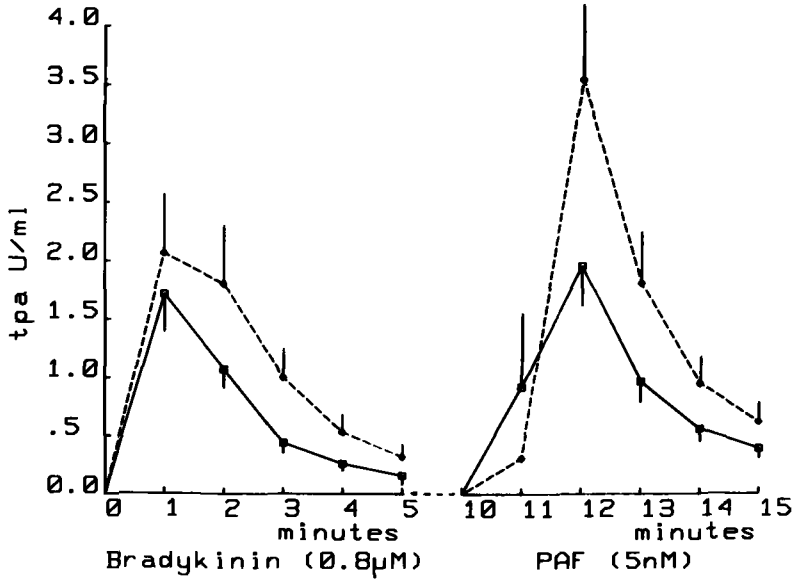

Fig. 1. Time course of the release of t-PA as induced by bradykinin (*0.8 µM*), followed - after a *5 min* washout - by PAF (5 nM). Data shown are means ± s.d. for five control perfusions (□), and three perfusions performed in the continued presence of 10 µM BL 194 (◇).

Table 2. Effect of pentoxifylline, BL 194 and HWA 448 on the induced release of vWF from rat hindlegs.

vWF (U/ml) (peak values of a 5 min perfusion)

Compound	Release induced by Bradykinin (0.8 µM)	Release induced by PAF (5 nM)	Number of experiments
Controls	0.59 ± 0.18*	0.61 ± 0.12	4
Pentoxifylline (10 µM)	0.60 ± 0.08	0.67 ± 0.14	3
BL 194 (10 µM)	0.43 ± 0.13	0.32 ± 0.05**	3
HWA 448 (10 µM)	0.60 ± 0.23	0.47 ± 0.07	3

* Mean ± s.d.

** Significantly different to controls by ANOVA ($p < 0.01$).

Although the effects of HWA 448 on both t-PA and vWF induced release were not found to be statistically significant, they did seem to follow the same trend as the effects seen by BL 194 on the PAF-induced release of both t-PA and vWF (Table 1 and 2).

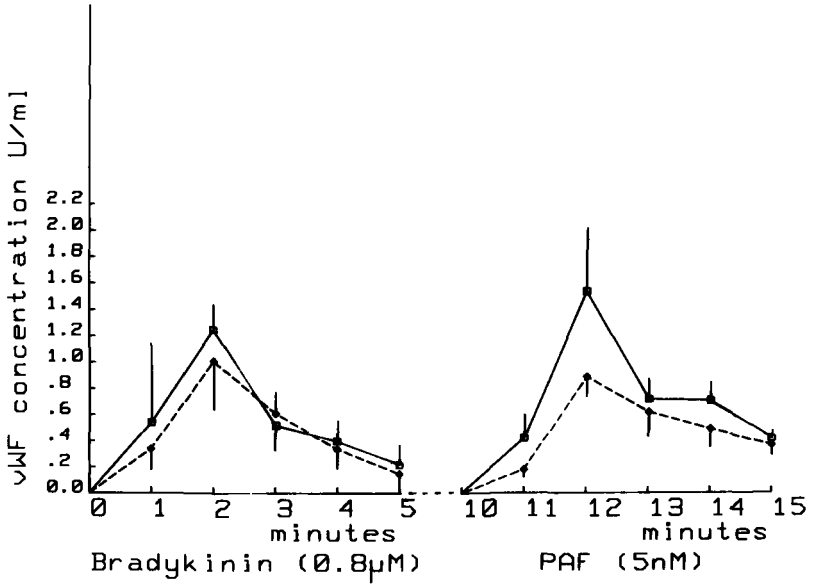

Fig. 2. Time course of the release of vWF as induced by bradykinin (0.8 μM), followed by PAF (5 nM). Data shown are means ± s.d. for four control perfusions (□) and four perfusions performed in the continued presence of 10 μM BL 194 (◇).

The effects of BL 194 and IBMX on t-PA and vWF induced release in the perfused hindleg.

As pentoxifylline and BL 194 have been reported to have PDE inhibitor activities, experiments investigating the actions of BL 194 and the (non-selective) phosphodiesterase inhibitor (PDE-1), IBMX were performed. IBMX (100 μM, ref. 25) was perfused both on its own and in combination with BL 194. IBMX itself did not induce any t-PA or vWF release, but it did potentiate the release of t-PA induced by both bradykinin and PAF (Table 3). BL 194 did not affect the t-PA release induced by bradykinin but again did increase that induced by PAF. The combination of BL 194 and IBMX had an additive effect on t-PA release by both bradykinin and PAF. Total sum values (data not shown) over a 5 minute perfusion period showed the same effects as the peak values in Table 3.

Similar to BL 194, the effect of IBMX on vWF release was again found to be opposite to its potentiation effect on t-PA release. IBMX reduced the release of vWF induced by

bradykinin while BL 194 reduced the release of vWF induced by PAF (Table 4, Fig. 2). However, *in contrast* to the results concerning t-PA release, no additive effect for vWF release could be demonstrated by the combination of IBMX and BL 194.

Table 3. Effect of IBMX and BL 194 on the induced release of t-PA from rat hindlegs.

t-PA release (U/ml) (peak values of a 5 min perfusion)

Compound	Release induced by Bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.03 ± 0.05*	1.89 ± 0.18	4
BL 194 (10 μM)	0.90 ± 0.16	2.28 ± 0.11	4
IBMX (100 μM)	1.56 ± 0.50	2.36 ± 0.29	4
BL 194 (10 μM) + IBMX (100 μM)	2.15 ± 0.67	2.99 ± 0.67	4

* Mean ± s.d.

For bradykinin, using two-way ANOVA IBMX significantly increased t-PA release ($p < 0.025$) but BL 194 did not.

For PAF, using two-way ANOVA, both IBMX and BL 194 were found to be significant in increasing t-PA release ($p < 0.025$).

Table 4. Effect of IBMX and BL 194 on the induced release of vWF from rat hindlegs.

vWF release (U/ml) (peak values of a 5 min perfusion)

Compound	Release induced by Bradykinin (0.8 μM)	Release induced by PAF (5 nM)	Number of experiments
Controls	1.25 ± 0.19*	1.54 ± 0.48	4
BL 194 (10 μM)	1.01 ± 0.37	0.89 ± 0.15	4
IBMX (100 μM)	0.62 ± 0.27	0.97 ± 0.29	4
BL 194 (10 μM) + IBMX (100 μM)	0.60 ± 0.20	0.96 ± 0.21	4

* Mean ± s.d.

For bradykinin, using two-way ANOVA IBMX was found to significantly decrease release ($p < 0.05$) but BL 194 did not.

For PAF, using two-way ANOVA, BL 194 was found to reduce release significant ($p < 0.025$).

The effects of pentoxifylline and BL 194 on *in vivo* fibrinolysis.

In the first set of experiments, endotoxin was injected into rats to induce an increase in PAI activity. Both pentoxifylline and BL 194 had no effect, either in increasing or in decreasing the induction of PAI activity by endotoxin (Table 5).

Table 5. Effect of pentoxifylline and BL 194 on induction of PAI activity by endotoxin.

Pretreatment	Plasma PAI activity U/ml
Saline	132 ± 45*
Pentoxifylline (20 mg/kg)	118 ± 18
BL 194 (20 mg/kg)	122 ± 31

* All data shown are mean ± s.d. (n = 5).
No significant differences in PAI activity by ANOVA.
Animals were bled three hours after the injection of endotoxin (10 μg/kg).

The sub-acute effect of pentoxifylline and BL 194 on baseline fibrinolytic parameters was next investigated by injecting rats daily for four days with these compounds. There was no significant effect seen on either the plasma PA activities or the PAI activity as a result of the pretreatment with either pentoxifylline or BL 194 (Table 6).

Table 6. Effect of sub-acute pretreatment on baseline levels of plasma PA and PAI activities.

Pretreatment	Plasma PA activities (U/ml)			Plasma PAI activity U/ml
	Total	t-PA	u-PA	
Saline (2.5 ml/kg)	2.14 ± 0.41*	0.47 ± 0.10	1.26 ± 0.27	6.9 ± 1.8
Pentoxifylline (50 mg/kg)	2.33 ± 0.21	0.35 ± 0.08	1.25 ± 0.05	5.4 ± 1.9
BL 194 (50 mg/kg)	2.08 ± 0.27	0.40 ± 0.12	1.24 ± 0.13	5.6 ± 0.5

* All data shown are mean ± s.d. (n = 5).
No significant difference seen by ANOVA in any parameter.

The effect of pentoxifylline and BL 194 on the PAF-induced PA release *in vivo* was also studied. Acute pretreatment with pentoxifylline and BL 194 (for one hour) had no effect on the PAF-induced PA release in the whole animal as measured in euglobulin plasma fractions (Table 7).

Table 7. Effects in plasma euglobulin fractions of pretreated rats on PA release (PAF (0.4 μ/kg) used to induce PA release).

Pretreatment	Number of experiments	% of controls		
		Total PA	t-PA	u-PA
Saline (2.5 ml/kg)	6	100 ± 12*	100 ± 13	100 ± 9
Pentoxifylline (50 mg/kg)	3	85 ± 10	77 ± 22	111 ± 28
BL 194 (50 mg/kg)	3	83 ± 12	74 ± 24	84 ± 9

* Mean ± s.d.
No significant differences were seen by ANOVA.

Similar investigations, using an acute pretreatment with pentoxifylline and BL 194, were performed on the induction of fibrinolytic activity in rats using dilute blood clot lysis times assay. The rats were injected with pentoxifylline (20 mg/kg), BL 194 (20 mg/kg) or saline one hour before the injection of PAF (0.5 μg/kg). The injection of PAF rapidly resulted in a large reduction of the dilute blood clot lysis times to the same extent in all the rats (Fig. 3; c.f. 26). There was no significant difference seen between the saline group or those pretreated with either pentoxifylline or BL 194 throughout the experiment.

DISCUSSION

Several reports have suggested that pentoxifylline has beneficial effects on the fibrinolytic system in animals and humans. Jarrett et al. (11) showed an improvement in fibrinolytic activity and lowered fibrinogen levels in humans taking pentoxifylline (200 mg three times daily over three months). Satewachin et al. (12) also showed an activation of fibrinolysis (the results of an increase of plasminogen activator concentration and a

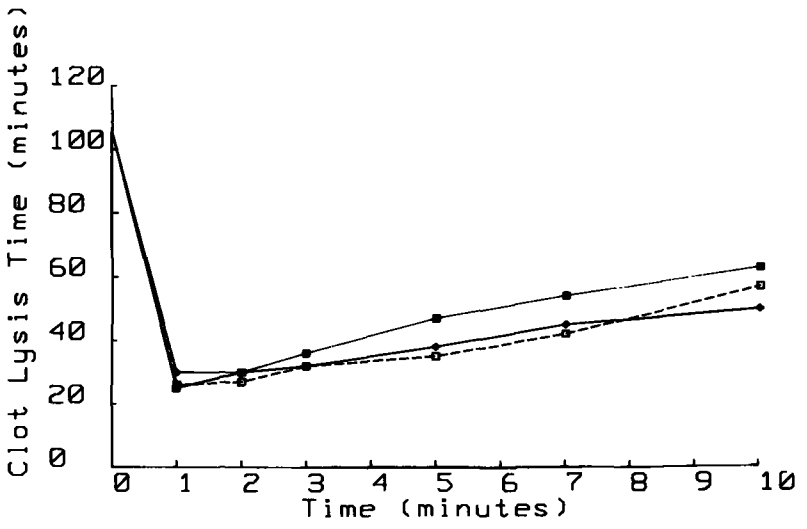

Fig. 3. Effect of PAF (0.5 μ/kg i.v.) on the dilute blood clot lysis times *in vivo* in control rats (□; n = 4) and in rats pretreated one hour earlier with pentoxifylline (20 mg/kg; ■; n = 3) or with BL 194 (20 mg/kg; ◊; n = 4). For reasons of clarity, mean values only are shown. At no time point was there a significant difference between the three groups by one-way ANOVA.

decrease in antiplasmin activity) in patients taking pentoxifylline. Ambrus et al. (27) found that pentoxifylline slightly increased thrombolysis in stumptailed monkeys and that it also potentiated the thrombolytic effect of u-PA and streptokinase-induced thrombolysis.

In 1987 Klöcking et al. published that BL 194, the first metabolite of pentoxifylline, directly induced PA release from the vascular wall in perfused pig ears. Our results, using the perfused rat hindleg model, do not confirm this finding. In contrast to the perfused pig ear (13), where release of PA is detectable in the absence of stimulation, no baseline release of PA occurs in our system. Pentoxifylline, BL 194 and HWA 448 alone did not induce any release of PA from the vascular wall of the rat hindleg. Our results did, however, show that BL 194, enhanced t-PA release induced by PAF and bradykinin (Table 1). It is thus likely that the increased release described by Klöcking et al. (13) in pig ears is due to enhancement, and not to induction, of release. In previous work we have shown that the release of t-PA and vWF are simultaneous and quantitatively correlated (23) using several different drugs and conditions. As BL 194 was found to potentiate t-PA induced release, we were expecting to see BL 194 also enhancing the

induced vWF release. To our surprise we encountered the opposite effect: BL 194 had an inhibitor effect on the amount of vWF released by PAF (Tables 2 and 4). The same opposing effects were seen when using IBMX, and HWA 448 also had the same tendency to increase t-PA and decrease vWF as did BL 194.

Several reports have suggested that pentoxifylline acts as a PDE-inhibitor and increases cAMP levels in various human (29) and animal tissues (30-32), including platelets (33). BL 194 has been shown to be a more potent PDE-I than pentoxifylline (Schönharting, 1989, personal communication). Our results are compatible with the involvement of such a PDE inhibitory effect for BL 194, as IBMX had (using PAF as an inducer) a similar effect to that of BL 194 on both t-PA release (increase), and vWF release (decrease).

The *in vivo* experiments involving pentoxifylline and BL 194 showed no increase in either t-PA or u-PA activity after either acute or sub-acute pretreatment of the animals with these drugs, with or without stimulation of PAF.

Recent findings suggest that pentoxifylline enhances PMN motility (8) and inhibits the inflammatory action of interleukin-1 (IL-1) and tumor necrosis factor, TNF (34-36). Endotoxin stimulates the production of IL-1 and TNF. The three compounds, endotoxin, IL-1, and TNF also stimulate production of PAI and procoagulatory activity in rats. Recently, it could be demonstrated that pentoxifylline reduced endotoxin-induced procoagulatory activity in rats, thus interfering with disseminated intravascular coagulation *in vivo* (Dickneite, 1989, personal communication). However, pentoxifylline and BL 194 had no effect on the induction of PAI in our experiments.

The mechanism of action of BL 194 in the perfused rat hindleg seems to be very complex and may involve several mechanisms, especially as BL 194 has no direct effect that can be explained as an agonist-receptor interaction, but acts by enhancing and reducing induced release of t-PA and vWF respectively. This dual modulating effect of BL 194 suggests a beneficial effect on thrombotic tendencies. The antithrombotic effects of pentoxifylline seen after chronic treatment of patients may be attributed to its first metabolite, BL 194 (37).

In summary, we have shown that BL 194, the first metabolite of pentoxifylline, enhances in perfused rat hindlegs the induced release of t-PA, and reduces the concomitant release of vWF. In our perfused system pentoxifylline itself is inactive, possibly due to a lack of metabolism of the compound.

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CHAPTER 9

SUMMARY AND CONCLUDING PERSPECTIVES

In Chapter one the two proteins, tissue-type plasminogen activator (t-PA) and von Willebrand factor (vWF), were briefly introduced and the possible pathways involved in their acute stimulated secretion (release) from the rat perfused hindleg endothelial cells were discussed. It was suggested that the release of t-PA from endothelial cellular stores is the most likely mechanism to explain the acutely increased levels of circulating t-PA. It had been shown that endothelial cells store vWF within the Weibel-Palade bodies, and it is this store of vWF that is released in response to a stimulus. A working hypothesis for the release of t-PA (or vWF) was suggested in Chapter one (Figure 1). Briefly, release would be induced by the interaction of an appropriate ligand with its endothelial cell receptor, leading to the activation of the phosphatidate-phospho-inositol pathway and/or G-transducing proteins, increased intracellular free calcium, calcium influx, enzyme activation (e.g., phospholipase activation, adenylate and guanylate cyclase activation), increased availability of arachidonic acid and arachidonic acid metabolites, activation of protein kinase C, finally resulting in the induction of the release reaction. As a model system to study these pathways the isolated perfused rat hindleg system was used (Chapter one, Figure 2).

In Chapter two a study was described to establish whether the t-PA released from perfused rat hindlegs was derived from stores of t-PA or from recently-synthesized t-PA. The release of t-PA was studied after inhibiting protein synthesis by cycloheximide for periods of 1 to 5 hours, and the effect of protein synthesis inhibition by cycloheximide on the t-PA content of hindleg skeletal muscle and lung was also studied. The results showed that continuing protein synthesis was not required for t-PA release to occur and that tissue levels of t-PA were little affected by prolonged inhibition of protein synthesis suggesting that the t-PA released from endothelial cells following stimulation is derived from a stable endothelial storage pool of t-PA.

It had been suggested by Emeis and Kluft (1985), based on a series of inhibitor studies, that products of the lipoxygenase pathway are involved in the acute release of t-PA from vessel walls. Chapter three shows that leukotrienes C_4 and D_4, though not other eicosanoids, can induce in rats the acute release of t-PA, both *in vivo* and in the perfused rat hindleg model.

In humans both t-PA and vWF are simultaneously released into the blood by various

stimuli, such as venous occlusion, exercise and infusion of adrenaline and 1-desamino-8-D-arginine vasopressine (DDAVP). Chapter 4 shows that in the experimental model of the perfused rat hindleg the induction of acute release of t-PA was also accompanied by release of vWF. The time-course of release was similar for both proteins, peaking at 1-2 minutes and decreasing to baseline levels over a 5 minute period, and the amounts of t-PA and vWF released by a compound were closely correlated. The acute release of both t-PA and vWF was found to be dependent on the presence of calcium in the perfusion buffer and A-23187, a calcium ionophore, was able to induce the release of both proteins into the perfusate fluid in a rapid acute fashion. The protein kinase C activator, PMA, also released t-PA and vWF but this release did not follow the usual time-course of release seen with other comppounds, but remained at a constant level for 15 minutes. The data presented in Chapter 4 indicate that the mechanism of acute release for both t-PA and vWF is very similar and could involve the same cellular pathways.

In Chapter five the involvement of the phospholipase pathway in the acute release of t-PA was investigated. The release of t-PA induced by a number of agents, was found to be inhibited by mepacrine, a phospholipase A_2 inhibitor and by nor-dihydroguaiaretic acid, a lipoxygenase inhibitor, suggesting an intermediary role for fatty acid lipoxygenation in t-PA release. The release of t-PA induced by PAF was also inhibited by cytochrome P-450 mono-oxygenase inhibitors such as metyrapone, SKF 525-A, and ketoconazole, indicating that the cytochrome P-450 mono-oxygenase pathway may be involved in the chain of reactions leading to the acute release of t-PA, Phospholipase A_2 did not induce any t-PA or vWF release in our system, however, phospholipase C (from Clostridium perfringens) did, implying a possible phospholipase C pathway in acute release. Pretreatment with pertussis toxin had no effect on the acute release of t-PA or vWF, it neither induced nor affected the induced release of both proteins.

The investigation in Chapter six established that the acute release of t-PA and vWF requires the presence of extracellular calcium and calcium influx into the endothelial cells. L-channel blockers were found to have little effect in our system, as did the calcium agonist BAY-K-8644, supporting the theory that voltage-operated calcium channels in endothelial cells are not involved in release. The release of t-PA and vWF may also be dependent on a calmodulin-dependent pathway in the cell.

The modulation of the induced acute release of t-PA and vWF by compounds affecting cyclic nucleotide levels was studied in Chapter seven. The intracellular concentrations of the cyclic nucleotides appear to be regulated by two classes of enzymes, the cyclase enzymes and the phosphodiesterases. Using drugs that stimulate or inhibit these classes

of enzymes, it was shown that cGMP down-regulates the acute release of both t-PA and vWF while the role of cAMP, if any, still remains poorly defined.

The effect on fibrinolytic components of pentoxifylline (Trental®, a clinically used drug to improve peripheral vascular circulation and to relieve intermittant claudication), its first metabolite, BL 194, and its analog HWA 448 were studied in rats (Chapter 8). *In vivo,* neither pentoxifylline nor BL 194 affected baseline levels of plasma t-PA and PA inhibitor activity. However, using the perfused rat hindleg system, BL 194, but not pentoxifylline or HWA 448, significantly enhanced the PAF-induced acute release of t-PA and, in contrast, reduced the simultaneous PAF-induced release of vWF. By its opposite effects on t-PA and vWF release, BL 194 might favourably influence the thrombotic balance.

In the introduction of this thesis we proposed a hypothesis (see Figure 1, Chapter 1) for the possible pathways involved in the acute release of t-PA and vWF. Different aspects of Figure 1 have been studied and to briefly conclude, the following points can be deduced based on the data provided in this thesis: t-PA like vWF can be acutely released from a stable cellular storage pool in endothelial cells; inhibiting the products of the phospholipase pathway, especially those of the arachidonic acid cascade, reduces the acute release of both t-PA and vWF; both extracellular calcium and calcium influx are necessary for the acute release of both t-PA and vWF; activation of the protein kinase C pathway results in little release; and finally regulation of this acute release involves the cyclic nucleotides, cGMP, and possibly cAMP.

In the above summary of the data provided in this thesis it is clear that though the perfused rat hindleg model is a very efficient example of a perfused vascular bed, and experiments performed on rats can be checked *in vivo* (such as PAF or the leukotriene induction of t-PA release) the information it provides is limited to an external overall view of endothelial cell functions (black box system). Using this system one can induce the acute release of t-PA or vWF, inhibit it, modulate it by various enzymes or products involved in the cellular mechanisms of release, but one cannot determine the exact intracellular mechanisms involved. The data provided in this thesis help to clarify the general intracellular mechanisms involved in this acute release. Further studies following the same general outline such as the involvement of the phospholipase pathway, the roles of calcium and the cyclic nucleotides in the acute release mechanism, should be conducted but using an *in vitro* endothelial cell model where the intracellular pathways may be simpler to identify and study than in an intact perfusion system such as the rat hindleg (Kooistra, 1990). However, it is the combination of *ex vivo, in vivo,* and *in vitro* data that is important in the elucidation of these complex pathways. That t-PA is found

in endothelial cells has been clearly established, however, the storage pool of t-PA in endothelial cells has yet to be identified. Further studies using an *in vitro* cell system might help to clarify this aspect of t-PA release, as it has been clarified for the presence and release of vWF from the Weibel-Palade bodies.

The work presented in this thesis should be used as a guideline to further investigate and elucidate the exact intracellular pathways involved in the acute release of t-PA and vWF from endothelial cells.

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SAMENVATTING

Hoofdstuk 1 geeft zowel een korte inleiding met betrekking tot twee eiwitten die in dit proefschrift centraal staan: weefsel-type plasminogeen activator (t-PA) en von Willebrand factor (vWF), als een indicatie hoe de acute, gestimuleerde secretie ("release") van deze twee eiwitten uit endotheelcellen zou kunnen verlopen. De voorgestelde hypothese houdt in dat secretie uit een intracellulaire voorraad het waarschijnlijkste mechanisme is om een snelle toename in de plasmaconcentraties van circulerend t-PA te verklaren. In ander onderzoek was al aangetoond dat vWF in endotheelcellen ligt opgeslagen in opslaggranula, de zgn Weibel-Palade lichaampjes, waaruit het in reactie op prikkeling van de cel wordt gesecreteerd. Vervolgens wordt in Hoofdstuk 1 een werkhypothese gegeven om de secretie van t-PA en van vWF te verklaren.

Kort samengevat stelt deze werkhypothese (zie figuur 1 in Hoofdstuk 1) dat de secretie van t-PA en vWF in gang wordt gezet door het binden van een ligand aan diens receptor op de plasmamembraan van de endotheelcel. Deze interactie leidt a) tot activering van G-eiwitten en de fosfatidaat-inositolfosfaat route, b) een toename van de intracellulaire calciumconcentratie, c) influx van calcium, d) activering van een aantal enzymen, waaronder fosfolipasen en adenylaat- en guanylaat-cyclase, e) een toename in beschikbaar arachidonzuur en arachidonzuurmetabolieten, en f) tot activering van protein kinase C. Waarna deze processen samen resulteren in de inductie van secretie van t-PA en vWF.
Het in dit proefschrift beschreven onderzoek heeft tot doel deze werkhypothese te toetsen. Als modelsysteem is voor dit onderzoek gebruik gemaakt van de geïsoleerd geperfundeerde achterpoot van de rat (figuur 2 in Hoofdstuk 1).

Hoofdstuk 2 beschrijft een onderzoek naar de rol van eiwitsynthese bij de secretie van t-PA en stelt de vraag: is het uit de geperfundeerde achterpoot van de rat uitgescheiden t-PA afkomstig van een in de cel opgeslagen liggende voorraad t-PA, of is het afkomstig van kort daarvoor gesynthetiseerd t-PA? De secretie van t-PA werd bestudeerd nadat de eiwitsynthese met behulp van de eiwitsyntheseremmer cycloheximide voor een periode van 1 tot 5 uur vrijwel volledig geremd was geweest. Ook werden zowel in de skeletspier van de achterpoot als in de long de t-PA concentraties gemeten. Uit de resultaten bleek dat ook zonder voortdurende eiwitsynthese t-PA secretie kon optreden en dat, als gedurende langere tijd de eiwitsynthese in deze weefsels werd geremd, dit vrijwel geen invloed had op de weefselniveau's van het t-PA. Al bij al suggereerden deze resultaten dat, bij acute

secretie van t-PA, het gesecerneerde t-PA afkomstig was uit stabiele voorraden t-PA in de weefsels.

Op grond van een reeks onderzoekingen met behulp van remmers was al door Emeis en Kluft (1985) gesuggereerd dat producten van een lipoxygenase route betrokken waren bij de acute secretie van t-PA uit vaatwandendotheel. In Hoofdstuk 3 van dit proefschrift wordt beschreven hoe leukotrieen C_4 en leukotrieen D_4 in staat bleken in de geperfundeerde achterpoot een acute secretie van t-PA te induceren, terwijl andere eicosanoïden (bijvoorbeeld de cyclooxygenase producten prostaglandine E_2 en prostacycline) daartoe niet in staat waren. De leukotriënen C_4 en D_4 induceerden ook *in vivo* een acute secretie van t-PA in de rat.

Bij mensen kan acute secretie van t-PA in het bloed worden gestimuleerd door procedures als veneuze stuwing en inspanning, of door stoffen als adrenaline of 1-desamino-8-D-arginine vasopressine (dDAVP). Ook de plasmaconcentratie van vWF neemt in deze gevallen sterk toe. In Hoofdstuk 4 wordt aangetoond dat in de geïsoleerd geperfundeerde achterpoot van de rat de acute secretie van t-PA eveneens gepaard gaat met acute secretie van vWF. Het verloop van de secretie in de tijd was voor beide eiwitten hetzelfde: een of twee minuten na het begin van de prikkeling trad maximale secretie op, waarna gedurende drie tot vier minuten de secretie afnam, zodat aan het eind van een vijf minuten durende prikkeling voor beide eiwitten weer vrijwel de basislijn was bereikt. Ook waren de gesecreteerde hoeveelheden t-PA en vWF sterk gecorreleerd. De acute secretie van zowel t-PA als vWF bleek afhankelijk van de aanwezigheid van calcium in de perfusievloeistof, terwijl de calciumionofoor A-23187 in staat was de secretie van t-PA en vWF sterk te stimuleren. Phorbol-myristaat-acetaat (PMA), een activator van protein kinase C, bleek bovendien enige secretie van t-PA en vWF te induceren, maar deze gestimuleerde secretie vertoonde een geheel ander tijdspatroon dan de door andere stimuli geïnduceerde secretie. De door PMA veroorzaakte secretie bleef gedurende 15 minuten op een stabiel, laag peil en vertoonde geen piekpatroon. De in Hoofdstuk 4 gepresenteerde gegevens duidden erop dat de bij de secretie van zowel t-PA als vWF betrokken mechanismen een sterke overeenkomst vertonen en mogelijk via dezelfde cellulaire wegen verlopen.

In Hoofdstuk 5 worden experimenten over de mogelijke rol van fosfolipasen en hun reactieprodukten bij de acute secretie van t-PA gepresenteerd. Secretie van t-PA werd geïnduceerd met een reeks van stoffen (bradykinine, substance P, thrombine, carbachol, calciumionofoor A-23187 en plaatjes-activerende factor). In al deze gevallen werd door mepacrine, een fosfolipaseremmer, en nor-dihydroguaiareetzuur (NDGA), een lipoxygenaseremmer, de secretie van t-PA vrijwel volledig geremd,

hetgeen erop duidde dat de lipoxygenatie van een vetzuur bij de secretie van t-PA een rol zou kunnen spelen. Daar BW755C de secretie niet remde, werd met behulp van de remmers metyrapon, SKF 525-A en ketoconazol bekeken of misschien ook de cytochroom P-450-afhankelijke mono-oxygenase route bij de secretie betrokken was. Daar deze drie remmers de secretie van t-PA (en vWF) sterk verminderden (en NDGA bovendien de mono-oxygenase route kan blokkeren) is het waarschijnlijk dat ook een cytochroom P-450-afhankelijke mono-oxygenatie bij de secretie betrokken is. Fosfolipase A_2 gaf in ons systeem geen secretie van t-PA of vWF, een fosfolipase C (uit Clostridium perfringens) echter wel, waaruit mogelijk kan worden afgeleid dat ook *in vivo* de fosfolipase C-route een rol speelt bij de acute release van t-PA. Voorbehandeling van ratten met Pertussis toxine had 24 uur later geen enkel effect op geïnduceerde secretie van vWF of t-PA; het is dan ook aannemelijk dat pertussis-gevoelige eiwitten geen rol van betekenis spelen bij acute secretie van t-PA en vWF.

Het in Hoofdstuk 6 beschreven onderzoek toonde aan dat acute release van t-PA en vWF afhankelijk was van de aanwezigheid van extracellulair calcium en calcium influx. Stoffen die calcium L-kanalen blokkeren hadden weinig effect, evenals de calcium agonist BAY-K-8644. Deze gegevens waren in overeenstemming met de theorie dat spanningsafhankelijke calciumkanalen niet betrokken zijn bij secretie uit endotheelcellen. Bij de secretie van t-PA en vWF leek daarentegen wel sprake te zijn van een calmoduline-afhankelijk proces.

Het effect van stoffen die de cellulaire niveaus van cyclische nucleotiden (cAMP en cGMP) beïnvloeden vormt het onderwerp van Hoofdstuk 7. Bij de regulatie van deze niveaus zijn twee klassen enzymen betrokken: de cyclasen en de fosfodiesterasen. Met behulp van farmaca die deze enzymen beïnvloeden, kon worden aangetoond dat cGMP een negatief effect had op de acute release van t-PA en vWF, terwijl over een mogelijke rol van cAMP geen duidelijkheid kon worden verkregen

Het laatste onderzoek (Hoofdstuk 8) betrof de effecten op fibrinolytische componenten in de rat van a) Pentoxifylline (Trental®, een geneesmiddel dat klinische toepassing vindt om de perifere circulatie te verbeteren en claudicatio intermittens te verlichten), b) zijn belangrijkste metaboliet BL 194, en c) een analoog (HWA 448). Pentoxifylline en BL 194 hadden *in vivo* geen effect op de basale plasmaniveaus van t-PA en PA remmer activiteit. In de geperfundeerde achterpoot had BL 194 daarentegen wel een effect, terwijl Pentoxifylline en HWA 448 het nog steeds lieten afweten. BL 194 verhoogde de door plaatjes-activerende factor geïnduceerde secretie van t-PA, maar verlaagde aan de andere kant de secretie van

vWF. Door deze tegengestelde effecten op de secretie van t-PA en vWF, zou BL 194 een gunstig effect kunnen hebben op de haemostatische balans.

In de inleiding van dit proefschrift (Hoofdstuk 1) werd een werkhypothese geformuleerd over de processen die een rol spelen bij de acute secretie van t-PA en vWF (zie figuur 1 in Hoofdstuk 1). Uit het in dit proefschrift beschreven onderzoek kunnen over deze processsen de volgende conclusies worden getrokken:

- zowel t-PA als vWF kunnen acuut worden gesecerneerd uit stabiele voorraden die in/op de endotheelcellen aanwezig zijn;
- het remmen van de vorming van produkten uit de fosfolipase route (in het bijzonder uit de arachidonzuurcascade) remt op zijn beurt sterk de acute secretie van t-PA en vWF;
- voor de acute secretie van t-PA en vWF zijn extracellulair calcium en calcium influx nodig;
- bij de regulatie van de acute secretie zijn zowel proteïn kinase C als de cyclische nucleotide cGMP - en mogelijk ook cAMP - betrokken.

Uit deze samenvatting moge blijken dat het model van de geïsoleerd geperfundeerde achterpoot van de rat zeer bruikbaar is voor het bestuderen van de secretie in een geperfundeerd vaatbed, en wel mede omdat de in dit modelsysteem verkregen gegevens (zoals de inductie van secretie door plaatjes-activerende factor of leukotriënen) *in vivo* kunnen worden gecontroleerd. De belangrijkste beperking van het model is echter dat men alleen informatie kan krijgen over de werking van het systeem als geheel (black box systeem). In dit model kan de secretie van t-PA en vWF door een grote verscheidenheid aan componenten worden geïnduceerd, geremd en gemoduleerd, maar kan men niet nauwkeurig meten wat er in de cel aan de hand is. De in dit proefschrift gepresenteerde gegevens zijn van belang voor een beter inzicht in de vraag welke processen bij de secretie van t-PA en vWF betrokken zijn. Om deze processen nauwkeuriger te kunnen bestuderen zal echter een ander modelsysteem moeten worden opgezet, waarbij men in eerste instantie kan denken aan een *in vitro* celsysteem waarbij de secretie van t-PA en vWF in gekweekt endotheel kan worden gemeten (Kooistra, 1990). Naar verwachting zullen in een dergelijk systeem de te onderzoeken routes (fosfolipasen, calcium, cAMP/cGMP) eenvoudiger toegankelijk en beter kwantificeerbaar zijn. Het is echter van het grootste belang dat men ook in de toekomst de *in vivo, ex vivo* en *in vitro* systemen blijft combineren voor het ophelderen van de complexe routes die bij de secretie door endotheelcellen betrokken zijn. Hoewel de aanwezigheid van t-PA in endotheelcellen duidelijk is vastgesteld, moet de manier waarop t-PA in de endotheelcel is opgeslagen

nog worden bepaald. Nader onderzoek aan *in vitro* celsystemen kan ook hier meer opheldering verschaffen, zoals dat ook voor vWF is gebeurd.

Het in dit proefschrift gepresenteerde onderzoek moge als leidraad dienen voor nadere studies naar de cellulaire routes die verantwoordelijk zijn voor de acute secretie van weefsel type plasminogeen activator en von Willebrand factor.

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CURRICULUM VITAE

The author of this thesis was born in Jerusalem on the 26th September 1957. She attended primary and secondary classes at the Anglican International Church School in Jerusalem. In 1974 she went to England to complete her studies for "O" and "A" levels. In 1979 after studying Pharmacy for a year she started a joint honours BSc degree course in Biochemistry and Physiology at Queen Elizabeth College, University of London. On completing this in 1982 she continued her studies and was awarded her MSc in Pharmacology in 1984 from Chelsea College, University of London. During her postgraduate years she taught undergraduate practical classes in pharmacology and worked on a project involving the bioassay of opioids at Chelsea College. In 1985 she married and came to the Netherlands where in March 1986 she started work at the Gaubius Institute under the supervision of Dr. J.J. Emeis. In September 1990 the work for this thesis was completed and for the present the author is still attached to the Gaubius Institute.