DIFFERENTIAL ACTIVATION AND INHIBITION OF HUMAN PLATELET SHAPE CHANGE, MICRO- AND MACROAGGREGATION, IN WHOLE BLOOD AND PLATELET-RICH PLASMA

by

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ABSTRACT

The relative sensitivities of adenosine diphosphate (ADP)-induced activation, and of Iloprost (ZK 36 374)-mediated inhibition were determined in autologous platelet-rich plasma (PRP) and freshly collected whole blood (WB) for the sequence of activation steps: unactivated platelets -> shape change (SC) -> early platelet recruitment (PA) -> macroaggegation (TA). Shape change was measured microscopically and turbidometrically, while PA and TA were measured by electronic particle counting and turbidometry respectively.

In PRP, the ADP sensitivity $[ADP]_{1/2}$, ([ADP] giving half maximal rate) was determined for the above activation sequence. Distinct $[ADP]_{1/2}$ values were obtained in PRP from log dose-response studies, with a relative dose dependency for SC, PA_3 and TA in the order of 1:3:~4. Differential inhibition in PRP of the above activation scheme was evaluated for ZK. IC_{50} values corresponding to ZK concentrations causing 50% inhibition of rates of TA (V_a), PA (PA_3) and SC (V_s) were found in the relative ratios of 1:~3:~5, when measured at a common ADP concentration for all three parameters, or 1:~2:~3 when determined at respective $[ADP]_{1/2}$ values for each parameter. Thus approximately 3-5 times more ZK is required to respectively inhibit the rates of shape change V_s and early platelet recruitment (PA_3), than that needed to inhibit the rate of turbidometrically measured macroaggregation (V_a).

In WB distinct ADP sensitivities ($[ADP]_{1/2}$) were obtained as above, with a relative dose dependency for SC and PA_3 in the order of 1:~2. IC_{50} values causing 50% inhibition of PA (PA_3) and SC (V_s) were found in the relative ratios of 1:~2.

In a parallel study, $[ADP]_{1/2}$ values for PA_3 in WB were shown to be comparable to, or in about half the observations ~2x greater than that measured for PRP. Independent of an individual donor's ADP sensitivity, IC_{50} values for PA_3 in WB were similar at all times to values determined in PRP.

RESUME

Les sensibilités relatives de l'activation induite par de l'ADP et de l'inhibition mediée par l'Iloprost (ZK 36374) ont été determinées pour le plasma agité, citraté et riche en plaquettes (PRP) et pour du sang complet fraichement recueilli, pour la sequence d'activation suivante: plaquettes au repos -> changement de forme (SC) -> petites aggregation des plaquettes sanguines (AP) -> macroaggregation de plaquettes (TA). Les vitesses initiales du changement de forme des plaquettes sont estimées selon deux méthodes: (1) la pente de la diminution initiale de la transmission de la lumière à travers du PRP, et (2) l'examen directe de la morphologie des plaquettes par microscopie à contraste de phase.

Dans le PRP, la sensibilité de l'ADP, (la concentration d'ADP requise pour le demi-maximum du taux de changement) a été déterminée pour la sequence d'activation mentionné auparavant. Les valeurs pour $[ADP]_{1/2}$ ont été obtenues pour la sequence d'activation avec une dose de dépendence relative pour SC, l'AP et TA dans l'ordre de magnitude de 1:3:~4. L'inhibition differentielle du schéma d'activation a été evalué pour ZK pour le PRP. Des valeurs pour IC_{50}, qui correspondent aux concentrations de ZK causant 50% d'inhibition des taux maximum de TA (V_a), AP (AP_3) et SC (V_s), ont été trouvées dans des ratios rélatifs de 1:~3:~5, lorsque elles ont été mesurées pour une concentration d'ADP commune pour chaque paramètre; ou 1:~2:~3 lorsque les concentrations d'ADP correspondait à l'$[ADP]_{1/2}$ réspectivement pour chaque paramètre. Donc, aproximativement 3-5 fois ou 2-3 fois plus de ZK est requis pour inhiber respectivement les taux de changement de forme

(V_s) et de l'AP_3 que de la macroaggregation.

Dans le sang complèt, les valeurs $[ADP]_{1/2}$ furent obtenues comme ci-haut, avec une dose de dépendence relative pour le changement de forme des plaquettes et l'AP_3 dans l'ordre de 1:~2. Les valeurs IC_{50} causant 50% d'inhibition de l'AP (AP_3) et SC (V_s) par le ZK furent trouvées dans des ratios relatifs de 1:~2.

Dans une étude parallèle, la comparison des valeurs $[ADP]_{1/2}$ pour l'AP_3 dans le sang complèt, avec celles mesurées dans le PRP, se révèlent semblables ou bien, dans approximativement un demi des observations, ~2x plus grandes. Les valeurs IC_{50} pour l'AP_3 dans le sang complèt sont semblables en tout temps aux valeurs déterminées dans le PRP, indépendamment de la sensibilité de l'ADP pour un donneur individuel.

TO MY FAMILY

MOM, DAD, HOWARD AND STACEY

Thank you for all the encouragement, guidance and support which you have given me. I am greatly indebted, for this thesis would not have been possible without your inspiration and motivation.

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PUBLICATIONS ARISING FROM THIS RESEARCH

Published Abstracts

1) Pedvis, L.G., Wong, T., Frojmovic, M.M. Differential inhibition of Platelet activation by a stable prostacyclin analogue, Iloprost. CFBS. 29:PA172, 1986. Oral presentation at the Twenty-Ninth annual meeting of the Canadian Federation of Biological Societies, University of Guelph, 1986.

2) Pedvis, L.G., Wong, H., Wylie, J., Frojmovic, M.M. Differential inhibition of the platelet activation sequence: shape change, micro- and macro- aggregation, by a stable prostacyclin analogue (Iloprost). Thromb. Haemost. 58:652, 1987. XIth International Congress on Thrombosis and Haemostasis, Brussels, Belgium, July, 1987. Presented at the Platelet Inhibitors Poster Session.

Papers

1) Pedvis, L.G., Wong, T., Frojmovic, M.M. Differential inhibition of the platelet activation sequence: shape change, micro- and macro- aggregation, by a stable prostacyclin analogue (Iloprost). Thromb. Haemost. 59:323-328, 1988.

2) Pedvis, L.G., Wong, T., Frojmovic, M.M. Differential activation and inhibition of human platelets: Direct comparison of freshly-collected citrated whole blood and platelet-rich plasma. To be submitted to Thrombosis and Haemostasis, in condensed form.

PREFACE

In writing this thesis, the author has taken advantage of the option provided by the regulations of the Faculty of Graduate Studies and Research which allows for the inclusion as part of the thesis the text of original papers suitable for submission to learned journals for publication.

Chapter II has been published in Thrombosis and Haemostasis 1988; 59: 323-328. A condensed version of Chapter III is being submitted for publication. A full introduction with joint summaries and conclusions are included.

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ABBREVIATIONS

AA	arachidonic acid
ABP	actin-binding protein
ADP	adenosine diphosphate
BSS	Bernard Soulier Syndrome
cAMP	cyclic adenosine -3',5'-mono-phosphate
DAG	1,2-diacylglycerol
DE	discoechinocyte
DMS	demarcation system
DTS	dense tubular system
EDRF	endothelial-derived relaxing factor
EDTA	ethylenediamine-tetraacetic acid
FGN	fibrinogen
FBS	fibrinogen binding site
GP	glycoprotein
G-protein	guanine nucleotide-binding protein
GTP	guanylate triphosphate
IP_3	inositol 1,4,5-triphosphate
MK	megakaryocyte
OCS	open canalicular system
PA	platelet microaggregation
PI	phosphatidylinositol
PGD_2	prostaglandin D_2
PGE_1	prostaglandin E_1
PGI_2	prostaglandin I_2
PGDF	platelet-derived growth factor
PGX	prostaglandin X
PLA_2	phospholipase A_2
PLC	phospholipase C
PPP	platelet-poor plasma
PRP	platelet-rich plasma
r_p	axial ratio (thickness/diameter)
SC	shape change
SE	spheroechinocyte
%T	percent light transmittance
TA	macroaggregation
TA-1	primary, reversible platelet macroaggregation
TA-2	secondary, stable platelet macroaggregation
TEM	transmission electron microscopy
TPA	12-O-tetradecanoyl phorbol 13-acetate
TSP	thrombospondin
TXA_2	thromboxane A_2
V_A	maximal rate of platelet aggregation (from %T)
V_S	maximal rate of platelet shape change (from %T)
vWD	von Willebrand disease
vWF	von Willebrand factor
WB	whole blood
ZK	(ZK 36 374) Iloprost

Chapter I

General Introduction

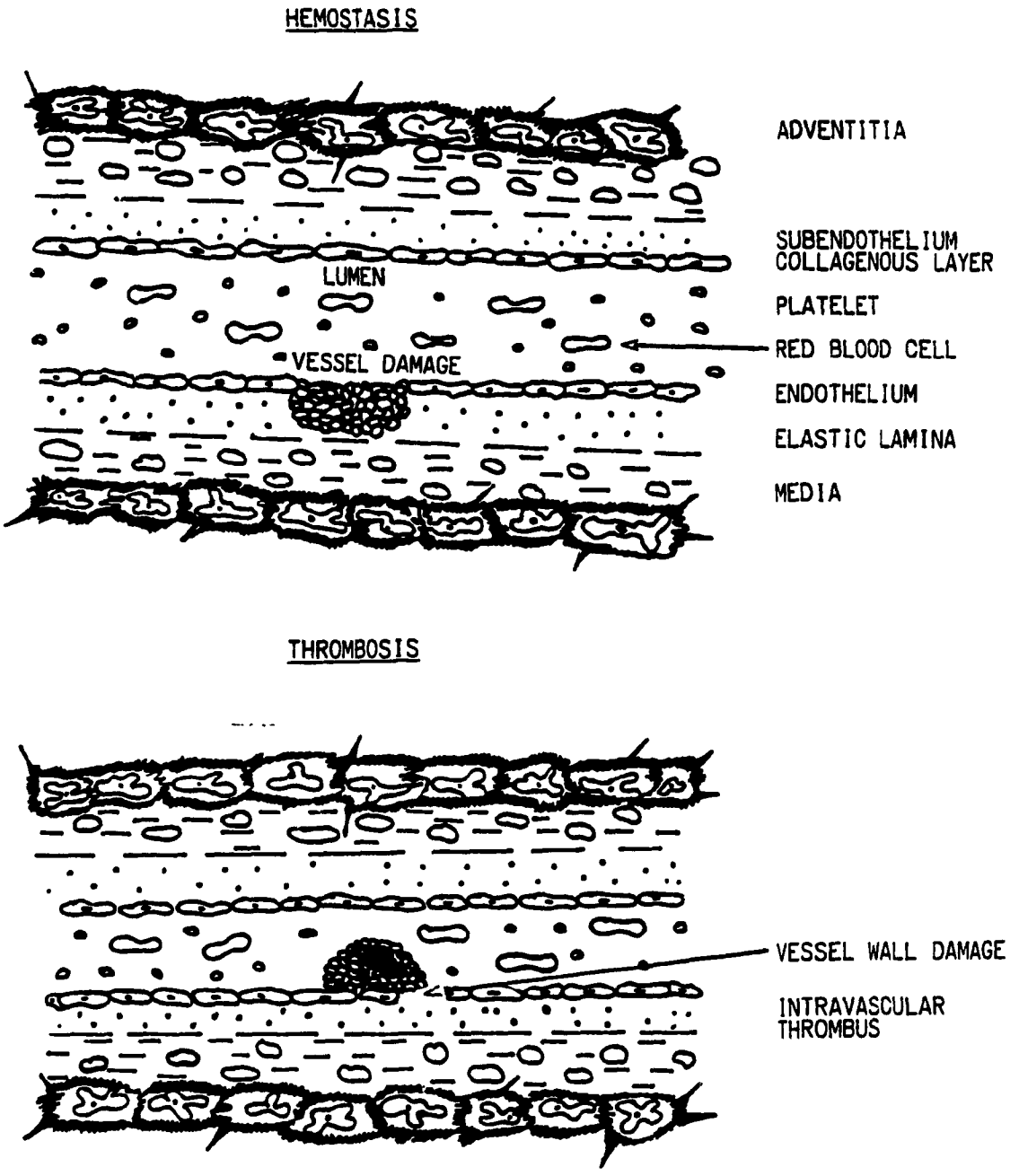
Platelet Structure and Function in Hemostasis

In the absence of any trauma in the human circulation, platelets circulate in the blood for 8-10 days as smooth disc-shaped cells that are non-adherent to each other or to healthy vascular endothelium. Therefore, it appears that the aqueous and cellular phases of blood are 'biocompatible' with the vessel, and cellular deposits are not generally observed on healthy, intact endothelial cells that line mammalian blood vessels.

Formation of a hemostatic plug at sites of vascular injury requires the participation of blood platelets. The hemostatic process represents a physiological defense mechanism designed to arrest bleeding from vessels that have undergone a break in their integrity. The process is rapid and localized without compromising fluidity of the blood in circulation. This mechanism takes place extravascularly as it occurs with exposure of the subendothelial matrix following vessel wall endothelial cell to cell or cell to subendothelial matrix separation. Hemostasis involves a complex integrated interaction of 1) blood vessel, 2) platelets, and 3) coagulation cascade to form a localized stable mechanical seal that subsequently undergoes slow removal by 4) fibrinolysis. Rapid, localized hemostasis within a fluid medium is achieved by complicated systems of activation and inhibition whereby excessive bleeding and unwanted thrombosis are minimized.

Thrombosis, however, takes place intravascularly and, therefore, can impede blood flow within the blood vessel rather than preventing escape of blood out of the vessel. Figure 1 clearly illustrates hemostasis versus thrombosis. While hemostatic plug formation is always initiated by vessel damage, the initiating stimulus for thrombosis may

Figure 1: Hemostasis versus Thrombosis



involve intravascular activation of the cellular and/or aqueous phase of blood. Normal hemostasis begins with a single unactivated platelet adhering to the subendothelial matrix, leading to its activation. The cell undergoes biochemical and physical changes which culminate in the release of adenosine diphosphate (ADP) from its dense granules. ADP activates subsequent cells arriving at the site of injury; resulting in the formation of microaggregates that normally leads to a regulated build-up of platelet macroaggregates. These macroaggregates may build-up to such an extent as to 1) impede local blood flow and/or, 2) create emboli that fragment off the main plug that could cause life-threatening thrombo-embolic complications seen, for example in pulmonary embolism and coronary artery disease.

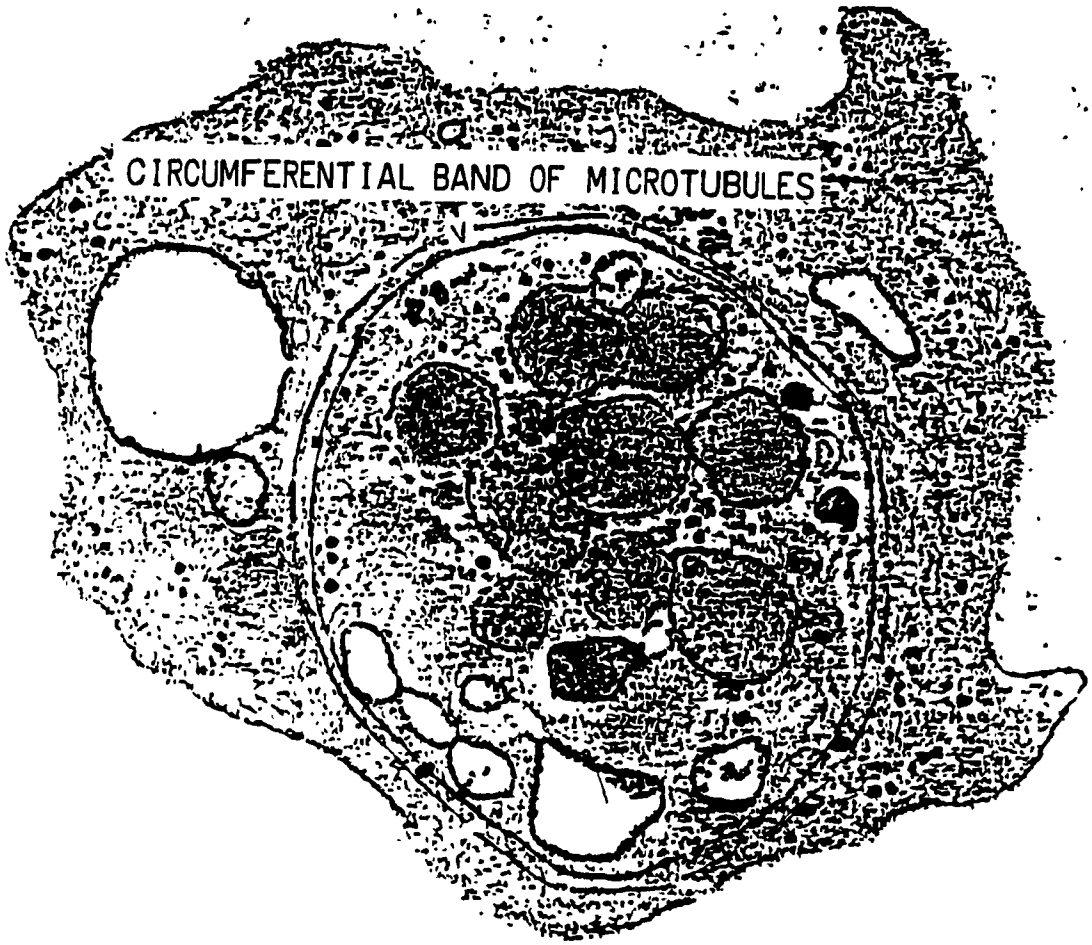
The interactions between blood cells are greatly influenced by the varying flow patterns in the vasculature. Platelets are subject to flow regimes with shear rates varying from 0 in bulk flow near the center of blood vessels, in separated flow, and in stasis in small arteries in the microcirculation, to greater than 1,000 sec^{-1} in arterioles and veins [1].

I. Platelet Ultrastructure

Regardless of the production site, human platelets circulate as anuclear, cytoplasmic discs with an average diameter of 3 to 4 uM, thickness of ~< 1 uM and volume of 10 fl. Platelet size distribution is very broad compared with other blood cells. In the non-stimulated state the discoid shape is maintained by a cytoskeleton of microtubules (Figure 2).

Membrane glycoprotein receptors mediate 1) shape change, 2) adhesion (sticking of platelets to surfaces), 3)

Figure 2: Platelet from a sample of citrated PRP exposed to the activator adenosine diphosphate (ADP) and fixed shortly after undergoing aggregation. The cell has lost its discoid form and the cytoplasmic organelles have become concentrated in a central region, surrounded by a circumferential band of microtubules.



internal contraction and secretion and 4) aggregation (cell to cell contacts). Platelet activation can lead to rearrangements of the membrane phospholipids to generate platelet procoagulant activity; chemical transformations of phospholipids can yield arachidonic acid (AA) and platelet active metabolites [2]. The surface membrane is continuous with a sponge-like, open canalicular system, and interdigitates with the dense tubular system which is not surface-connected. Channels of the open canalicular system and dense tubular system in platelets form interwoven membrane complexes morphologically identical to the association of transverse tubules and sarcotubules in embryonic muscle cells. This dual membrane system appears to constitute the calcium-regulating mechanism. Submembranous filaments and cytoplasmic filaments of the sol-gel zone constitute the contractile system of the platelet. Platelets contain substantial quantities of muscle proteins including actin, myosin, tropomyosin, alpha-actinin, actin-binding protein, filamin and troponin [2].

Energy for contraction is derived by aerobic metabolism in the mitochondria and anaerobic glycolysis utilizing glycogen granule stores. Three types of storage granules are present in platelets: 1) alpha granules, the most numerous, containing platelet-specific proteins (platelet factor 4, thrombospondin (TSP), B-thromboglobulin, platelet-derived growth factor (PDGF)) and proteins also found in plasma (fibronectin, albumin, fibrinogen, and coagulation factors V and VIII); 2) dense bodies, containing storage adenosine diphosphate (ADP), serotonin, calcium, and phosphates; and 3) lysosomal vesicles. Secretion involves the release of constituents from the storage granules into the open canalicular system.

Platelets are extremely sensitive cells and may respond to minimal stimulation (e.g. 0.1 uM ADP) by forming pseudopods that are important for cell to cell contacts [3].

A somewhat stronger stimulus causes platelets to become reversibly sticky with only partial loss of discoid shape (discoechinocytes (DE)). Thus, unactivated platelets (discocytes) are converted to discoechinocytes containing 1-3 pseudopods per platelet within 8-10 seconds, with increases in cell volume of up to 40%. This is followed by spheroechinocyte (SE) formation, consisting of roughly spherical platelets, detectable by 8-10 seconds after activator addition [4-6]. Platelet shape change (DE and SE formation) is triggered by an increase in the level of cytoplasmic calcium [4-6]. Extrusion of storage granules contents requires internal contraction.

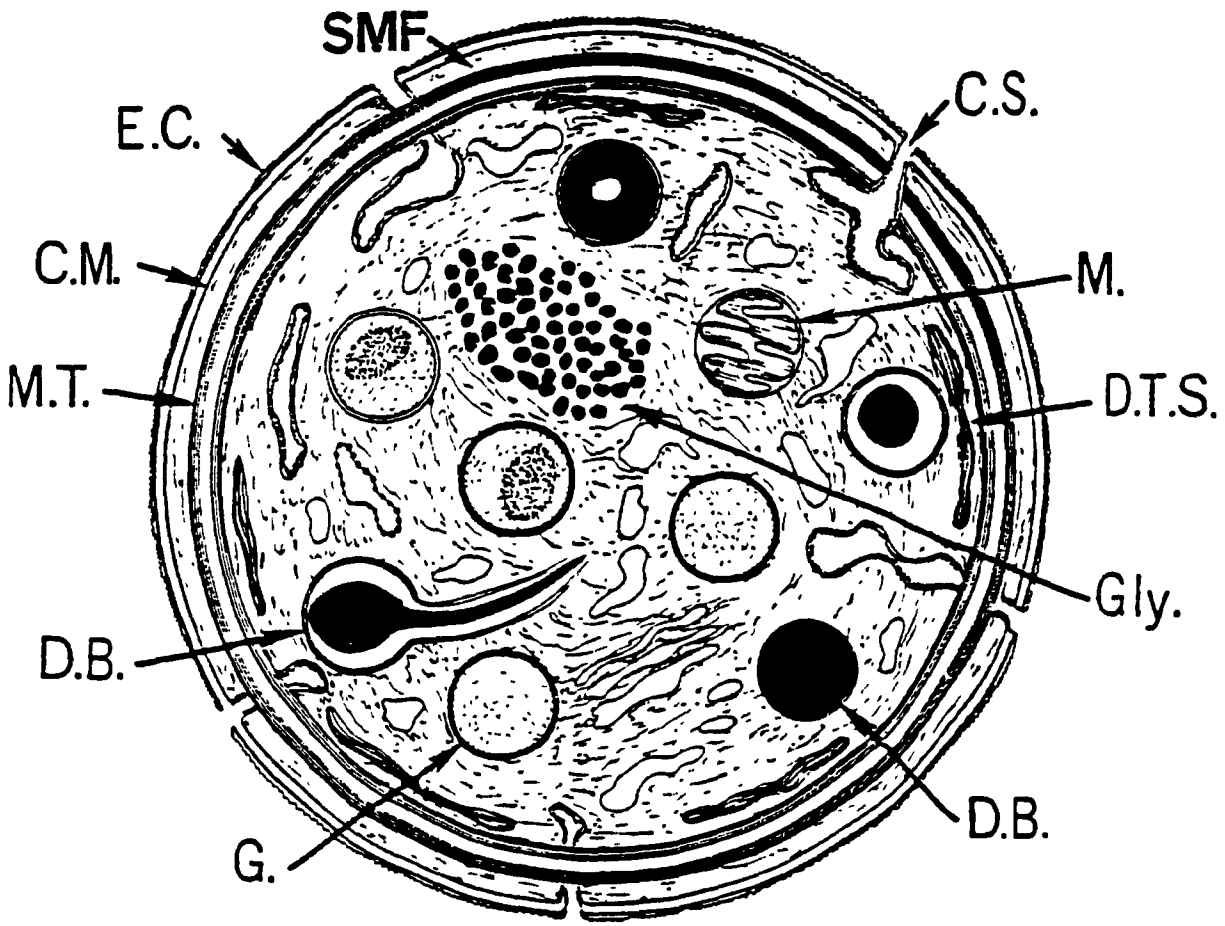
Following stimulation and in association with the contraction of the actomyosin complex, platelet microtubules undergo a concentric central shift with an inner clustering of organelles. The release reaction is the secretory process which involves discharge of constituents from the alpha, dense and lysosomal granules into the open canalicular system (OCS; see Figure 2). The alpha granules contain the adhesive (glyco) proteins which are thrombospondin (TSP), von Willebrand factor (vWF), fibrinogen (FGN) and fibronectin. TSP and FGN assemble on the platelet membrane and stabilize the secondary irreversible phase of aggregation, whereas vWF and fibronectin are responsible for platelet adhesion to surfaces. Dense granules contain ADP and serotonin which serve to further activate surrounding discocytes. High concentrations of thrombin or collagen cause the release of proteolytic enzymes from lysosomal granules which cause irreversible destruction of affected platelets.

It is evident that cellular integrity and organization is required for adequate platelet function [3]. The anatomy of platelets has been divided into four major regions (zones) in order to simplify the structural features and relate them to functional and biochemical activities [7,8].

The ultrastructure of the platelet is represented in Figure 3. The peripheral zone consists of the plasma membrane and closely associated structures making up the surface of the platelet and walls of the tortuous channels of the surface-connected open canalicular system (OCS). The OCS represents an expanded reactive surface to which plasma hemostatic factors are selectively adsorbed. An exterior coat or glycocalyx, rich in glycoproteins, constitutes the outermost covering of the peripheral zone. This covering, is clearly different from that exposed on the surfaces of red blood cells and leukocytes. Platelets contain a heavy thicker glycocalyx that is more dense than the surface coats of most other cells. The plasma membrane provides the receptors for stimuli triggering platelet activation and the substrates for adhesion-aggregation reactions. The middle layer of the peripheral zone is a typical unit membrane that is rich in assymetrically distributed phospholipids that provide the essential surface for interaction with coagulant proteins [9]. During activation there exists a change in the phospholipid distribution in the membrane, so that the negatively charged phosphatidylserine and phosphatidylinositol become available at the platelet surface. This transposition has been termed flip-flop [9].

The sol-gel zone is the matrix of the platelet cytoplasm. It consists of two fiber systems 1) the circumferential band of microtubules and 2) microfilaments, in various stages of polymerization that support the discoid shape of resting platelets and provide a contractile system involved in shape change, membrane-bound pseudopod extension, internal contraction and secretion [9]. In advanced stages of platelet activation, the bundle of microtubules becomes constricted in a tight-ring around centrally clumped organelles. The marginal bundle of microtubules appears to play a role in governing the

Figure 3: Diagramatic representation of a platelet in cross section as it appears in thin sections by electron microscopy. (White, J.G., Clawson, C.C., Gerrard, J.M. Platelet ultrastructure. In: Haemostasis and Thrombosis, Churchill Livingstone, New York, 1981).



internal contraction of platelets so that organelles are moved to the cell center, facilitating the secretory response. If secretion does not take place, the aggregated platelets recover their discoid form, with the return of the ring of tubules to its position under the cell wall. The result is the dispersion of the organelles to random positions within the cytoplasm.

Microfilaments constitute the second system of fibers in the platelet sol-gel zone, consisting of both actin and myosin, both crucially involved in cell contraction. Pseudopod formation is closely linked to this contractile process; however, it is regulated by special proteins which foster assembly of gelled actin into parallel filaments near the cell wall. Actin-binding protein (ABP) causes purified solutions of actin to gel and cross link. When alpha-actinin is added with ABP, the actin filaments not only gel, but form parallel associations typical of those that develop in platelet pseudopods. Pseudopod formation may favor early microaggregation by 1) increasing the collision frequency, due to increased effective platelet collision diameter [10], 2) minimizing electrostatic repulsions [11], and 3) providing a structural framework for intercellular membrane spreading for stabilization of adhesive contacts [12,13].

The organelle zone consists of alpha granules, dense bodies, lysosomes, and mitochondria randomly dispersed in the cytoplasm of the unactivated platelet. It serves in metabolic processes and for the storage of enzymes, nonmetabolic adenine nucleotides, serotonin, a variety of protein constituents, and calcium.

Membrane systems in platelets constitute the fourth "zone". The dense tubular system (DTS) has been shown to be a calcium sequestration site, important for triggering contractile events [14,15]. It is also the site where enzymes involved in prostaglandin synthesis are located (i.e., cyclo-oxygenase and thromboxane synthetase) [16-18].

II. Platelet Heterogeneity

Platelets are produced by fragmentation of megakaryocytes [19]. Megakaryocytes have varying ploidy. Studies with rat platelets suggest that 8n, 16n and 32n megakaryocytes produce large, medium and small platelets, respectively [20]. These size-dependent platelet subpopulations were shown to contain distinct types and amounts of membrane and enzyme systems. Large platelets were more dense, and had the most abundant mitochondria and secretory granules; while small platelets had the lowest densities and the most abundant internal membranes such as surface-connecting plasma membrane and dense tubular system including associated enzymes of the prostanoid pathway [20]. Haver and Gear separated human platelets by functional fractionation by removing the more reactive platelets forming aggregates at low activator concentrations; they found that reactive platelets were larger than unreacted platelets [21]. They observed that these functionally more active (larger) platelets, are also metabolically more active, possess a higher negative surface charge (e.g. for contact reactions), and may be a younger population than the smaller, reactive platelets [21]. Thus, a more recent study has shown that the largest human platelets (~15% of the total population) have been found to be about two times more quickly recruited into microaggregates and approximately two times more sensitive to aggregating agents such as ADP when compared to the smallest platelets (~15% of the total population) [22]. A number of other studies have also suggested that large platelets are more active than small ones, as measured by in vitro tests of platelet function [23].

The role played by platelet size and age in determining platelet function is a controversial issue [24]. Because

platelets have been reported to deteriorate in functional ability with both decreasing size [25] and increasing age [26], it has been suggested that size and age are dependant determinants of platelet activity. However, one study has reported size and age to be independent variables [27].

III. Platelet Production

A) Megakaryocyte Maturation

In recent years, new information has advanced our understanding of the processes underlying megakaryocyte maturation and platelet production. Platelets are produced by a fragmentation of the entire cytoplasm of a giant cell with a multilobulated nucleus. These cells designated megakaryocytes by Howell in 1890, constitute less than 1% of bone marrow cells [28].

Acccording to commonly accepted schema, a pluripotent stem cell, with the capacity to differentiate into multiple haematopoietic lineages, becomes progressively restricted in this differentiative capacity such that its progeny give rise to only a few lineages.

These comitted cells are capable of undergoing many divisions before being able to increase their size by endoreduplication. This cell proliferates into diploid megakaryocytic precursors, which at a later stage lose the capacity for cell division and acquire the ability for endoreduplication of DNA. It has been confirmed that DNA replication occurs only in young megakaryocytes possessing some granules and demarcation membranes [29]. At varied ploidy levels (8-64) DNA replication stops and cells greatly increase the production of granules and demarcation membranes. Therefore, before any evidence of cytoplasmic differentiation occurs, the cell synthesizes all the DNA

that it ultimately will carry. The synthesis stops at the earliest sign of cytoplasmic differentiation. The nature of the mechanism that induces the ceasing of DNA replication is unknown [28]. However, it has been postulated that the appearance, in the cytoplasm, of the contractile protein, thrombosthenin, may trigger a message to stop DNA synthesis [30]. At this stage after the cell has synthesized all its DNA, it is now termed a polyploid cell. The polyploid cells increase in cell volume until the point of recognition as megakaryocytes (MK). The recognizable megakaryocytes undergo further cytoplasmic maturation into platelet-forming cells [31].

The final cell size and the amount of cytoplasm are determined by the DNA content. Thus the number of platelets produced by megakaryocytes depends on this ploidy value [32].

B) Platelet Liberation

Platelet liberation from megakaryocytes was first described by Wright (1910) and since his report the mechanism of platelet liberation has persistently been of controversy.

Researchers have suggested various modes of platelet release. Some observed that whole megakaryocytes (or proplatelets) were simultaneously fragmented into platelets in the pulmonary circulation. Others described that individual platelets were liberated one after another from the pseudopods of the megakaryocyte [33]. While still others believed that the DMS in the mature MK subdivided small cytoplasmic areas (platelet zones) each of which developed into platelets.

Several authors have interpreted the presence of blebs or small protrusions on the MK surface to represent the

development of platelets which are destined to be released by a budding mechanism [33]. In this mode of platelet separation, the megakaryocytes projects large cytoplasmic pseudopods into the sinus or postsinal venule lumen; and later, platelets detach from these pseudopods one after another. By this means of platelet liberation the DMS does not seem to play a major role in platelet release [34]. In fact megakaryocytes lacking a DMS have been shown to release platelets in vitro [35]. In culture, rounded megakaryocytes were observed to possess these small blebs; however, in agreement with an earlier study by Thiery and Bessis [36], there was no evidence from time-lapse observations that these were released as platelets into the sinusoids. It has been suggested that the formation of these bleb-like structures may be attributed to a surface reorganization resulting from the transfer of megakaryocytes to an in vitro environment, or to a fixation artifact [33].

The dynamic process of platelets being formed this way has never been observed in vivo, although apparent disintegration of megakaryocytes into cytoplasmic fragments in both the sinusoidal and extrasinusoidal spaces does occur [37].

This has been tested more recently by Trowbridge et al., [38] who have suggested that platelets are produced in the pulmonary circulation by a physical fragmentation of megakaryocyte cytoplasm. Proplatelets released from the sinusoids can also undergo cytoplasmic fragmentation in the alveolar network.

Electron microscopy studies have indeed shown whole megakaryocytes and large megakaryocyte fragments (proplatelets), apparently in the process of migration across the parasinusoidal membrane into extracellular spaces [30]. These findings are compatible with a number of studies that have reported the presence of circulating megakaryocytes and proplatelets, possessing plentiful

cytoplasm in central venous blood, and the pulmonary circulation.

The pulmonary network acts as a filter for the circulating megakaryocytes and cytoplasmic fragments. In order that these fragments, which are much larger than the pulmonary arterioles and capillaries, can continue circulating, further fragmentation must occur in the lungs. The result is the platelet population. Megakaryocytes and proplatelets are therefore trapped by the pulmonary circulation, and complete their development in the alveolar capillaries, in response to both physical [39] and chemical stimuli e.g. prostacyclin [40].

Another mode of platelet formation and liberation is through the formation of a demarcation system; whereby, the cytoplasmic maturation of the MK is associated with the development of an extensive membranous system. In his transmission electron microscopy (TEM) study on mouse spleen megakaryocytes, Yamada [41] first observed that the earliest manifestation of the demarcation system (DMS) was the appearance of vesicles in the intermediate zone of the cytoplasm. He showed that these vesicles developed into a maze-like structure by coalescence. The DMS in mature megakaryocytes, separates numerous platelet zones throughout the intermediate zone of the MK cytoplasm. This system demarcates platelet 'zones' or 'territories' by enclosing and defining parts of the MK cytoplasm that would, in the end, be platelets. In this way the DMS forms the cell membrane of nascent platelets.

The origin of the DMS has variably been attributed to the MK cell membrane, endoplasmic reticulum, golgi system, and membranogenic areas of the cytoplasm. Current evidence indicates that none of the intracellular membrane systems are the origin of the DMS. After some dispute regarding the origin of the demarcation membrane system, Behnke described that the DMS was indeed derived from the megakaryocyte cell

membrane [29]. The invagination of the cell membrane, gives rise to the formation in the cytoplasm of scaffolding cylindrical-tubular structures; the cisternae of which are in communication with the extracellular space. Thereby, as the MK matures the DMS is continuously formed by an invagination process, whereby the MK membrane invaginates resulting in membrane-wrapped tubules whose cisternae are in communication with extracellular space. These tubular structures whose cisternae are in direct communication with extracellular space are subsequently transformed by a process of fusion-fission into flat sheets. It is these flat sheets of DMS which form the cell membrane of nascent platelets.

IV. Platelet Relation to Endothelium

One of the basic functional characteristics of intact, normal endothelium is its non-reactivity to platelets, leukocytes, and the coagulation factors. The thrombo-resistant character of the endothelium involves both passive and active mechanisms [42]. The endothelial proteoglycans, primarily heparan sulfate, provide a surface that is passively non-thrombogenic [43]. Active thrombo-resistance of the endothelium is achieved through several mechanisms including the 1) synthesis and release of prostacyclin (PGI_2) [44]; 2) synthesis and release of nitric oxide or endothelial-derived relaxing factor (EDRF)[44]; 3) secretion of plasminogen activators; 4) degradation of proaggregatory ADP by membrane-associated ADPase; 5) uptake, inactivation, and degradation of proaggregatory vasoactive amines; 6) uptake, inactivation, and clearance of thrombin; and 7) contribution of the cofactor (thrombomodulin) in the thrombin-dependent activation of protein C and the resultant destruction of coagulation factors V and VIII and the

release of plasminogen activators [45-51].

The normal endothelium acts as a natural barrier preventing thrombus formation on the vessel wall, and prevents the constituents of the blood from interacting with the subendothelial structures [52]. Moncada et al.,[45] postulated that the formation of prostacyclin by the cells lining the vessel wall is responsible for preventing platelet adherence to normal endothelium. However, direct testing of this hypothesis by inhibiting prostacyclin formation through the administration of aspirin to experimental animals has shown that platelets do not adhere when its formation is interrupted.

Prostacyclin (PGI_2) is a labile prostaglandin that potently inhibits platelet adhesion and aggregation. Modulation of PGI_2 production by injury factors including activated clotting enzymes serves to limit locally any hemostatic response [53]. The capacity of the endothelial lining to regulate PGI_2 production contributes to the nonthrombogenic properties of intact vascular endothelium. Endothelial denudation results in a loss of the non-thrombogenic surface as well as exposure of subendothelial connective structures to circulating blood.

The endothelium produces its own underlying connective tissue composed of several classes of collagen, proteoglycans, elastin and microfibrils [52]. This connective tissue matrix modulates the permeability of the inner vessel wall and provides the principal stimulus to thrombosis following vessel injury.

V. Platelet Membrane Glycoproteins, Adhesive Proteins and their Role in Aggregation

The initial event in hemostasis in response to vascular injury involves the adhesion of platelets to exposed

vascular subendothelium. The adherent platelets provide a cohesive surface for the buildup of an on-site platelet aggregate (primary hemostatic plug). Released tissue factor and the activated platelet surface provide acceleration of localized coagulation, resulting in the eventual stabilization of the platelet plug by fibrin strands. It is now apparent that these processes in hemostasis are mediated by specific membrane glycoproteins (GP) on the platelet surface.

Biochemical studies have shown that the exterior glycocalyx contains carbohydrate-rich domains of more than 30 membrane glycoproteins [54]. Since glycoproteins are predominant on the plasma face of the platelet membrane, they are presumed to be involved in most of the specific receptor and transport processes that involve the platelet surface. These include receptors for platelet stimuli (adenosine diphosphate, collagen, thrombin, adrenaline), platelet antagonists (prostaglandins D_2, E_2, and I_2), transport proteins (e.g., for serotonin), and specific receptors for Factor VIII, Factor Va, and thrombin, which localize and accelerate the final enzymatic reactions of the coagulation cascade. Other properties and functions of these membrane GP's include the following: 1) mediating platelet adhesion and aggregation, 2) participating in recognition phenomena and phagocytosis, 3) binding complement, 4) giving the platelet its antigenic specificity, and 5) stabilizing the platelet surface and controlling its surface charge [55]. The most abundant GP's are Ia, Ib, IIb, IIIa, IV and V. Glycoproteins serve to link surface-bound substrates with the intracellular contractile system.

Platelets adhere to collagenous fibrils, amorphous basement membrane-like material, and the elastin-like microfibrils. While adhesion to collagen may be important at low shear, it was noticed about 10 years ago that the

principle mechanism of platelet adhesion included a plasma and subendothelium protein von Willebrand factor (vWF) and a platelet membrane component GPIb. Platelets attach to exposed endothelium via GPIb, which serves as a surface receptor for vWF, which can bind to collagen or other components of subendothelium to mediate platelet adhesion [55].

Platelet adhesion to exposed vascular subendothelium is proportional to subendothelial bound vWF, and has been shown to be inhibited by heterologous and monoclonal antibodies against vWF. Over the last 2-3 years recent work has confirmed the important role of platelet membrane glycoprotein Ib in vWF-dependent platelet adhesion.

Initial evidence that GPIb played an important role in platelet adhesion came from studies on the Bernard-Soulier syndrome (BSS). In BSS patients there is a deficiency or an abnormality in GPIb and their platelets are found to have a decreased adhesion to subendothelium [56]. Similarly, in von Willebrand's disease there is a deficiency or abnormality of plasma vWF and, as in BSS, there is decreased platelet adhesion to subendothelium. Chemical cross-linking studies have confirmed that thrombin does, indeed, bind to GPIb on platelets [57]. Studies with monoclonal antibodies to GPIb inhibit thrombin-induced platelet aggregation and secretion [58].

The glycoprotein IIb-IIIa complex appears to be the most predominant glycoprotein on the human platelet plasma membrane. It is an intrinsic GP (~50,000 copies of each per platelet) distributed as a calcium-dependent heterodimer complexes [55,59] that can be solubilized by detergents. These GPIIb-IIIa complexes are found in the membrane of unstimulated platelets, and seem to be evenly distributed over the membrane [55,60]. About two-thirds of the complexes are randomly dispersed on the platelet surface, while the remainder are found between membranes of the SCCS

and the granules. The complexes are transmembrane and may interact with actin filaments within the platelet under stimulus conditions [55,60].

The GPIIb-IIIa complex has been shown to serve as the platelet receptor for fibrinogen, and also for other plasma proteins, including fibronectin and vWF, which contain the RGD (arg-gly-asp) amino acid sequence [55,59,60]. This complex undergoes conformational changes on the membrane surface following platelet activation to acquire the ability to bind fibrinogen. Previous studies of the GPIIb-IIIa fibrinogen receptor have been carried out on surface-activated platelets, and have shown that the fibrinogen-gold labels (FGN/Au) will not bind to platelets until significant shape change has occured [61-64]. This correlates well with findings of classical radiolabelled ligand binding studies, which indicate that fibrinogen does not bind with any specificity to an unactivated population of platelets [65,66]. Monoclonal antibodies directed against the transformed GPIIb-IIIa complex (e.g. PAC1), inhibit fibrinogen binding [67].

The results of these previous studies suggest that the shape change component of platelet activation may be a prerequisite for fibrinogen binding by acting to remove steric hindrance caused by other platelet membrane glycoproteins [64]. Fibrinogen binding may also be closely associated with cytoskeletal changes coincident with shape change, which may direct a reorganization (clustering) of the receptor in the plane of the membrane [55,61].

Initial evidence that transformed GPIIb-IIIa complexes constitute the platelet receptor for fibrinogen came from studies of platelets from patients with Glanzmann's thrombasthenia, which have a deficiency or abnormality of platelet membrane GPIIb-IIIa. Two calcium binding sites must be saturated with calcium prior to the firm adhesion of fibrinogen to the GPIIb-IIIa receptor [55]. There is also

evidence to suggest that FGN, when bound to GPIIb-IIIa, interacts with thrombospondin (TSP) in adhesive reactions [68]. TSP is an alpha granule protein that is secreted upon platelet activation and is believed to have an affinity towards GPIV [55]. The interaction with TSP appears to stabilize FGN binding to GPIIb-IIIa, thus strengthening platelet-platelet linkage. Other studies have since shown that GPIIb-IIIa complexes bind not only fibrinogen, but also vWF and fibronectin [69]. The GpIIb-IIIa complex is important for aggregation, since monoclonal antibodies directed towards this receptor result in a thrombasthenic-like state [70]. The failure of platelets from thrombasthenic patients to aggregate appears to be linked to the absence of GPIIb-IIIa, and thus the failure of thrombasthenic platelets to bind fibrinogen in response to physiological activators [70].

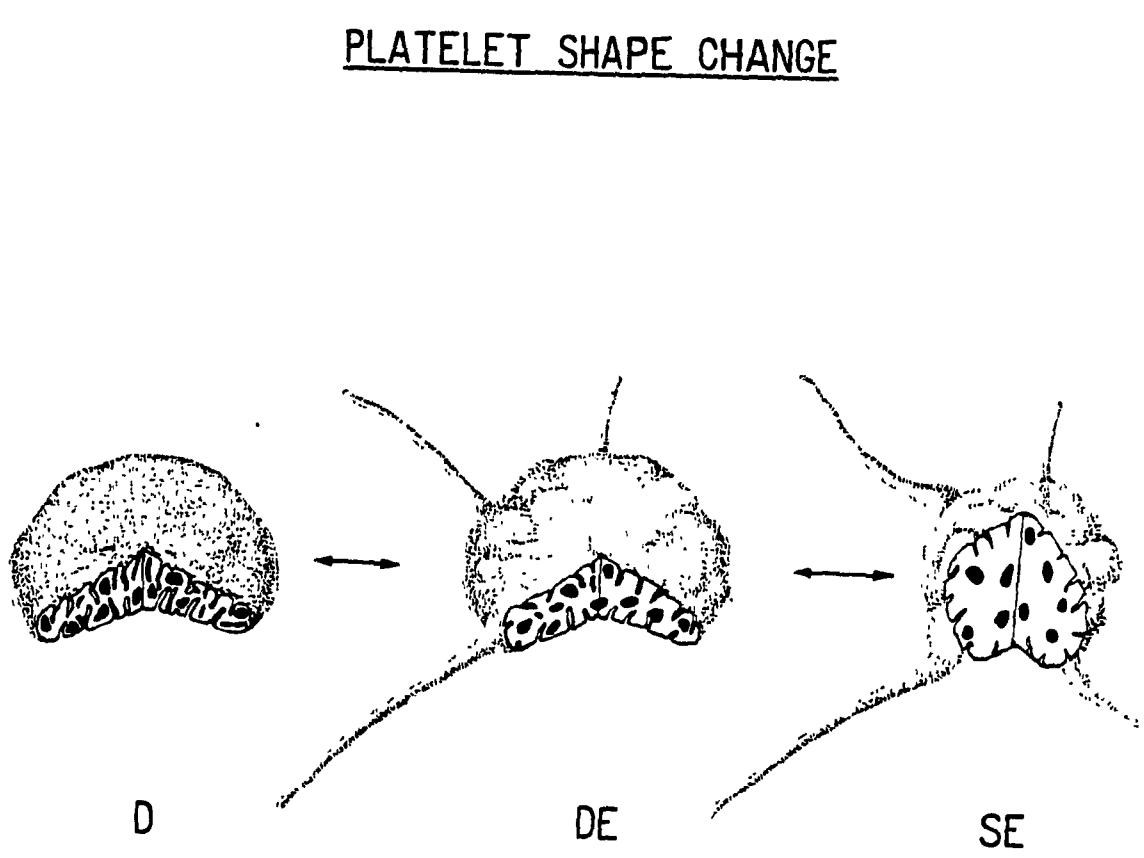
VI. Platelet Shape Change

In contrast to leukocytes which are covered with villous projections, platelets in the quiescent state appear as smooth-surfaced oblate spheroids. This is in contrast to the biconcave red blood cell.

In response to many stimuli, platelet morphology changes from that of a smooth, discoid cell to more spherical forms possessing pseudopods i.e., a more activated state (echinocyte). Figure 4 shows the structure of platelets in the reversible discocyte-echinocyte transformation. The transient hypervolumetric shape change is believed to be accompanied by an increase in externalized plasma membrane surface area [71], with the most probable source being the surface connected canalicular system [71].

Shape change is defined as any morphological alteration in the platelet as evidenced by changes in volume (size) or

Figure 4: 3-D structure of platelets in the reversible discocyte-echinocyte (D <-> DE <-> SE) transformation. (Frojmovic, M.M., Milton, J.G., J. Physiol. Reviews 62: 185, 1982: drawing modified by T. Wong).



in the topology of the total plasma membrane. Shape change occurs following the interaction of agonists such as ADP with receptors situated on the cell surface. The morphological changes are due to biochemical and physical mechanisms in the platelet that alter its shape. The extent of platelet shape change depends on the activator type, strength and time elapsed following activation.

Under phase contrast microscopy 1) edge-on discocytes (D) appear as smooth ellipsoids (the axial ratio = r_p < 0.5); whereas, face-on they appear circular with a dark center and 2) sphero-echinocytes (SE) appear circular with a white center in all orientations (r_p > 0.9) and 3) disco-echinocytes (DE) are all those platelets which do not satisfy the criteria for D and SE [71].

Platelet shape change (SC) has been described kinetically by aggregometric measurements of initial decreases in turbidity of platelet suspensions following addition of activators such as adenosine diphosphate (ADP) [3,72]. This parameter of platelet function has also been studied by direct particle observation using cine-microscopic analysis of single platelet shape, size and recruitment [3,71,72]. Although changes in platelet axial ratio (r_p), mean volume, refractive index and pseudopod formation all contribute to changes in %T with time, the initial decrease in % light transmission (%T) following activation, has been shown to reflect shape change and associated r_p [73].

Unactivated discocytes are converted to disco-echinocytes containing 1-4 pseudopods per platelet within 8-10 seconds following the addition of activator; with increases in cell volume of ~40%. This is followed by sphero-echinocyte formation, consisting of roughly spherical platelets, detectable by 8-10 seconds after activator addition, maximally formed by 15 seconds, and 20% smaller in volume than the original discocyte [4-6,10,71].

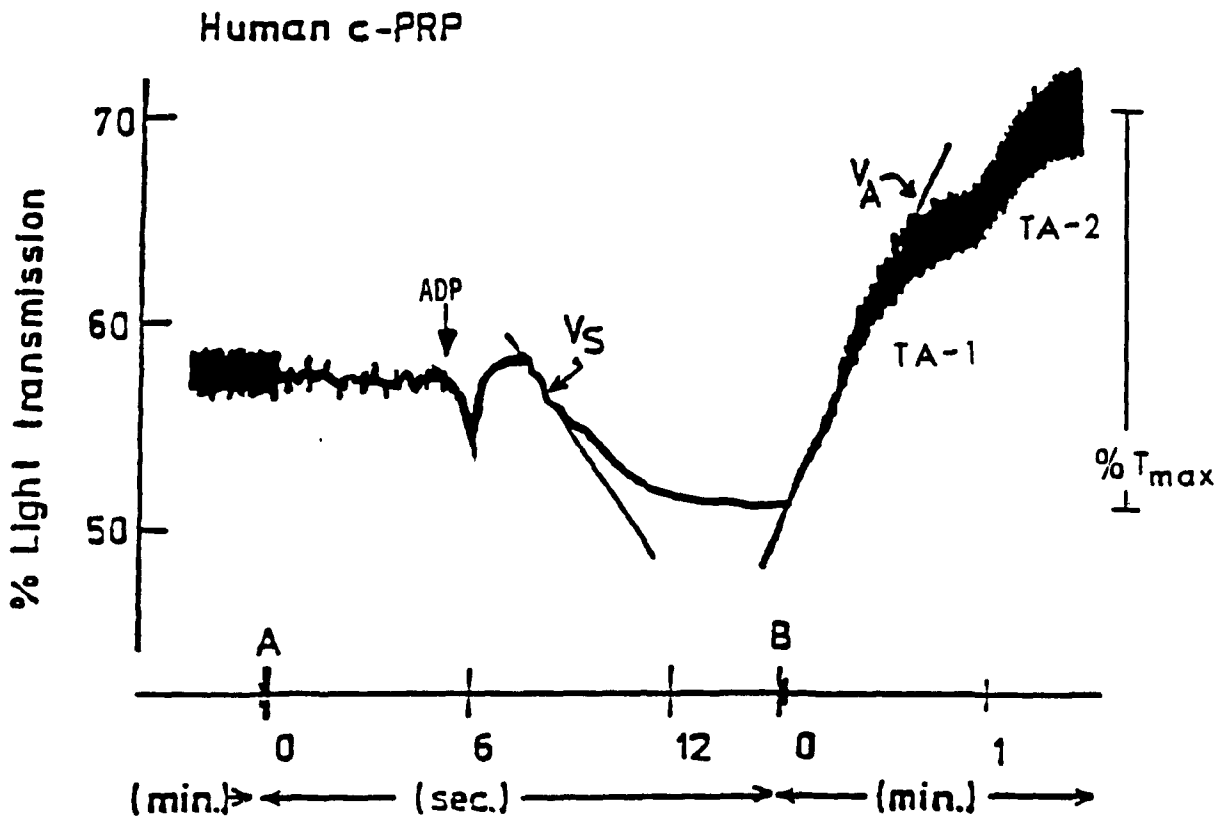
VII. Platelet Aggregation

Vessel disruption not only induces platelet adhesion but also initiates a series of complex and interdependent reactions including: 1) the release of dense granule ADP from adherent platelets; 2) the formation of small amounts of thrombin; and 3) the activation of platelet membrane phospholipase activity to generate thromboxane A_2. Release of ADP, thrombin formation, and thromboxane A_2 generation act in concert to recruit platelets from the circulation to produce the initial hemostatic plug.

The measurement of platelet aggregation has played a major role in the development of the current understanding of platelet function. This is due in part to the assumed primary relationship of in vitro aggregation and in vivo platelet function, and also because aggregation can be measured fairly easily. In 1962, both Born [74] and O'Brien [75] described a simple photometric method for measuring platelet aggregation. The addition of a platelet agonist to a stirred suspension of platelets results in the formation of platelet aggregates and an increase in light transmission through the opalescent platelet suspension (Figure 5). Small concentrations of an agonist such as thrombin may induce only shape change (which can be reversible) manifested by a sudden narrowing and small deflection in the baseline aggregometry tracing. Such changes reflect platelet shape change.

Shape change is the first event in stimulus-response coupling in platelet activation. It, like aggregation, requires agonist-receptor occupancy in order to maintain morphological and biochemical alterations to the platelet. Previous studies using ADP as activator have shown shape change to be most sensitive to activators and the most resistant to inhibitors, whereas the reverse holds true with

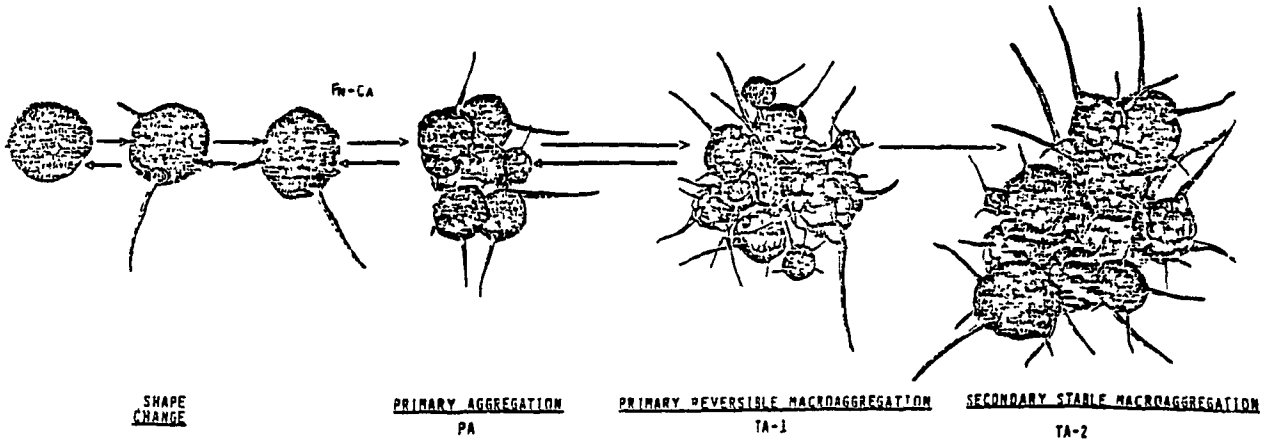
Figure 5: Aggregometry tracing showing ADP-induced platelet shape change and aggregation in human citrated PRP. The changes in the percent light transmission accompanying platelet shape change and aggregation were continuously recorded as a function of time. (Tang, S.S., Frojmovic, M.M. J. Lab. Clin. Med. 95: 241, 1980: drawing modified by T. Wong).



respect to aggregation [76]. With low concentrations of activator (ADP), the platelets will undergo shape change and activation. Along with extracellular calcium and fibrinogen, this leads to platelet microaggregation (PA) i.e., the formation of doublets and multiplets containing < 10-20 platelets per aggregate (Figure 6). Low activator concentrations (e.g., <1uM ADP) and low laminar flow conditions ($G < 30\text{-}60\ sec^{-1}$) lead to the formation of large visible (> 0.1 mm in size) macroaggregates which are mechanically fragile and revert back to platelet microaggregates with a very modest increase in shear rates ($G > 100\ sec^{-1}$). So microaggregation (PA) can proceed to form large reversible macroaggregates (TA-1), under these low activator conditions, when approaching very disturbed flow conditions (i.e., trapped flow). These flow regimes are found in situations where there exists Y-junctions, anneurisms, and eddy formation. So, under normal flow and low concentrations of agonist, platelets will only undergo early recruitment (microaggregation) [71]. Higher activator concentrations (e.g., ~ 1-3 uM ADP) at higher shear rates also yield turbidometrically visible macroaggregates (TA-1) which spontaneously revert to microaggregates with time [71,77]. With even higher stimulus (e.g., > 3-5 uM ADP), more binding sites are expressed thus further enhancing the stickiness of the aggregates and concomitant release reaction occurs, thus achieving secondary stable macroaggregation (TA-2) [71]. Upon release, thrombospondin (TSP) is secreted from the alpha granules. TSP by specifically interacting with fibrinogen, stabilizes these large aggregates. The binding sites for TSP appear to be different from the binding sites for the platelet membrane GPIIb-IIIa complex [68]. Indeed GPIV is now considered to be a distinct receptor for TSP [55].

The primary wave of aggregation (TA-1) reflects a loose platelet-platelet attachment. Primary aggregation is used

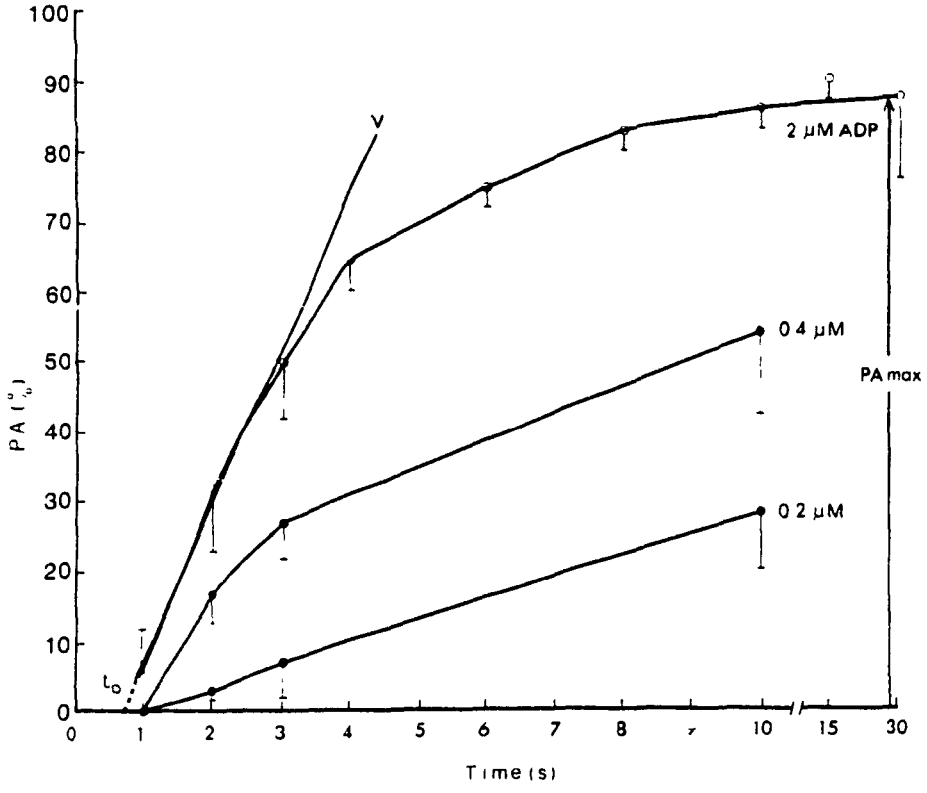
Figure 6: The sequence of platelet activation leading to irreversible macro-aggregation, consists of three main processes each having distinct activation requirements: shape change (SC), micro-aggregation (PA), and macro-aggregation (TA).



to refer to aggregation alone which can be reversible, as shown in the aggregometry tracing in Figure 5. The secondary or recruitment wave of aggregation accompanied by release (TA-2) occurs with higher concentrations of agonist and represents largely irreversible aggregation mediated by released ADP, thromboxane A_2 and thrombospondin (TSP). Even with biphasic aggregation essentially all platelets have aggregated during the initial phase while the second phase represents the consolidation into larger and more dense aggregates rather than recruitment of any more single platelets. Most platelets (> 75%) can form micro-aggregates containing from two to about eight platelets per aggregate prior to the detection of any increase in %T. In fact the increase in %T is detectable by ~6 sec, corresponding to the formation of aggregates containing > 7-10 platelets per aggregate [78]. The decrease in %T in the first 4-5 secs following activator addition quantitatively reflects changes in platelet shape and has been shown to be a measure of velocity of shape change, referred to as V_s [73]. This is followed by platelet aggregation which produces an increase in %T. The initial rate of %T increase is taken as a measure of the velocity of aggregation and is referred to as V_a [78]. The [activator] required to induce one-half maximal rate of SC (V_s) and TA (V_a), $[activator]_{1/2}$, was readily determined from a log dose-response plot of $V_s/(V_s)max$ or $V_a/(V_a)max$ versus activator concentration [76].

Platelet microaggregation (PA) is estimated from the percent decrease in the particle count determined with an electronic (resistive) particle counter [79]; or from singlet counting using a haemocytometer [78]. Figure 7 shows that the time course for platelet aggregation measured from the disappearance of single platelets (% platelet microaggregation) after addition of 2 uM ADP to stirred platelet-rich plasma (PRP) is S-shaped, with an

Figure 7: Kinetics of ADP-induced micro-aggregation (PA). (Frojmovic, M.M., Milton, J.G., Duchastel, A. J. Lab. Clin. Med. 101: 964-976, 1983).



approximately linear increase between 2 to 4 seconds [78]. Similar time courses have been reported for 0.2-10.0 uM ADP [78]. The maximum increase in % PA was typically attained at 8 to 10 seconds for 0.5 to 100 uM ADP and generally did not exceed 90% even when measured as late as 15 minutes after ADP addition. Early increase in % PA is expected to reflect early doublet to triplet formation, whereas % PA at 10 seconds generally reflects maximal disappearance of singlets, for the whole range of ADP concentrations studied (0.1-100 uM) [78].

The onset of platelet shape change precedes early recruitment of platelets by approximately 1 second. This does not reflect the mixing time of approximately 200 msec with the aggregometer [73]. The lag has been reported to reflect the time required for a calcium independent activation process [3].

VIII. Signal Transduction in the Blood Platelet: Phosphatidylinositol and Endoperoxide pathways

The behaviour of all cells from one instant to another is governed by signalling systems that translate external information into internal signals known as second messengers. Receptors on the cell surface function as molecular antennae, detecting external information (e.g., hormones, growth neurotransmitters or light) which is then transduced and amplified into second messengers which control many cellular processes such as metabolism, secretion, contraction, photo-transduction and cell growth. Activity in this field of signal transduction is growing very rapidly, because an imbalance of second messengers can cause a host of pathologies and clinical disorders including cancer and thrombosis [80].

The signalling system using cyclic adenosine

monophosphate (3'5'-cAMP) as its second messenger is the best known [81]. Until recently, we knew much less about the nature of the second messengers used by another major signalling pathway that utilizes the inositol lipids as part of its transduction mechanism.

In the action of a group of hormones, some neurotransmitters, and many other biologically active substances, signal-induced degradation of inositol phospholipids may generate important intracellular second messengers even though this species of phospholipids is a relatively minor component of mammalian cell membranes [80]. Nevertheless, important functions have been assigned to these such as receptor-mediated transmembrane signalling. The involvement of inositol phospholipids in the activation of a wide variety of cell surface receptors has a longer history than that of cAMP [82], being first suggested by Hokin and Hokin who showed that acetylcholine induces rapid breakdown and resynthesis of phosphatidylinositol (PI) in some secretory tissues such as the pancreas [83].

A) Membrane Receptors and Signal Transduction

Presently, the most favoured hypothesis about the mechanism of platelet activation by hormonal factors is that these agents, after interaction with their specific receptors induce the hydrolysis of membrane bound phosphoinositides by a phospholipase C action [84]. A characteristic feature of these receptors is that they are multifunctional in nature in that they have been implicated as part of a general transducing mechanism for the mobilization of calcium, for the activation of protein kinase C, for the release of arachidonic acid (AA) and for the activation of guanylate cyclase.

Although it is clear that the first event of stimulus response coupling is an interaction of the agonist with its

cell surface receptor, the mechanism by which the receptor-agonist complex initiates hydrolysis of inositol phospholipids is not clear. There is evidence that guanylate triphosphate (GTP) and its binding protein (G-protein) are involved in this receptor-mediated hydrolysis of inositol phospholipids in a manner analogous to the adenylate cyclase system [85,86].

Phospholipase C is specific for inositol phospholipids; with recent evidence indicating that PtdIns 4,5-P_2 is preferentially hydrolyzed compared with PtdIns 4-P and PI, especially at low concentrations of calcium [87]. The primary receptor stimulated event is probably the hydrolysis of PtdIns 4,5-P_2 to yield the apolar product DAG, and Inositol triphosphate (Ins-P_3; I(1,4,5)P_3) a water soluble product [88]. There are now many studies which support the notion that this hydrolysis of PtdIns 4,5-P_2 is a common response by many different kinds of cells to a wide variety of agonists [86]. The demonstration that the formation of Ins-P_3 precedes that of inositol monophosphate (the expected product of PI hydrolysis in Calliphora salivary glands) provides additional evidence that PtdIns 4,5-P_2 is the primary substrate for phospholipase C [89].

Occupation of a receptor by an agonist diverts PtdIns 4,5-P_2 out of its futile cycle towards phospholipase C by which it is cleaved to DAG and Ins-P_3. One unsolved problem in the formation of Ins-P_3 and DAG is that of how receptors are coupled to the PtdIns 4,5-P_2 phospholipase C. Agonists may induce a conformational change in the receptor, which in turn perturbs the membrane sufficiently to make PtdIns 4,5-P_2 accessible to the enzyme.

In many cells including platelets the earliest changes in inositol lipids appears to be a loss of PtdIns 4,5-P_2 with formation of DAG and Ins-P_3. An important point is that phospholipase C in intact platelets can be activated at basal intracellular calcium. One possible explanation is

that the enzyme, is biochemically dependent on calcium, but not physiologically regulated by the level of the divalent cation [90]. Other studies have shown the activation of phospholipase C to be independent of external calcium [91]. Agonists reported to cause breakdown of phosphoinositide via phospholipase C in human platelets include collagen, thrombin, vasopressin, adenosine diphosphate (ADP) and platelet-activating-factor. Thromboxane-A_2 (TXA_2) also appears to be an effective stimulus for inositol lipid breakdown and the effects of some agonists may be partly or largely attributable to prior formation of TXA_2 (see D) below: Model for Signal Transduction).

B) Physiological Roles of Second Messengers

a) Inositol (1,4,5) triphosphate (Ins-P_3)

Certain cells, such as the adrenal medulla, mast cells, and nerve cells seem to rely predominantly upon extracellular calcium, whereas others (e.g., liver, pancreas, parotid, blood platelets, anterior pituitary and certain smooth muscle cells) use intracellular calcium, especially during early periods of stimulation, in addition to extracellular calcium. However, the link between the activation of the surface receptor and the mobilization of calcium from intracellular (non-mitochondrial) reservoirs has been missing. The very rapid formation of inositol phosphates upon activation of the blood platelet with ADP suggested that Ins-P_3 may function as a second messenger to release internal calcium from the DTS [80]. Thus it is possible that Ins-P_3, one of the products of signal-mediated breakdown of PtdIns 4,5-P_2, may increase intracellular calcium levels (from 100 nM) within the platelet. Evidence for the Ins-P_3 calcium mobilizing hypothesis has been obtained by studying the effect of this second messenger on

various permeabilized cells; where Ins-P_3 could gain access to the non-mitochondrial intracellular calcium stores; this calcium releasing property of Ins-P_3 was first demonstrated in a preparation of rat pancreatic acinar cells permeabilized by incubation in a low calcium medium [92]. Ins-P_3 also induces the rapid formation of thromboxane B_2 in human platelets permeabilized with saponin [93]. Mobilized calcium activates a calmodulin dependent protein kinase which is responsible for the phosphorylation of a 20K protein (myosin light chain). Activation of this cytosolic protein leads to morphological changes in cell shape [81].

b) Diacylglycerol (DAG)

The second product of phosphoinositide hydrolysis, diacylglycerol (DAG), apparently acts by stimulating the calcium-activated, phospholipid-dependent protein kinase (protein kinase C) [94]. This enzyme can be rather directly activated by phorbol esters, such as 12-O-tetradecanoyl phorbol 13-acetate (TPA). At least one substrate of this protein kinase has been identified and purified in human platelets; a 47,000 MW protein [94]. The function of this protein is not yet known. The evidence linking its phosphorylation with the initiation of secretion is entirely based on the correlation of measured phosphorylation with measured secretion [94].

Protein kinase C is not active under basal conditions, requiring calcium as well as phosphoserine for its activation. If however, DAG is produced, it dramatically increases the affinity of protein kinase C for calcium, thereby rendering it fully active without a measurable increase in the basal intracellular calcium concentration (<100 uM) [95].

c) Other Inositide Metabolites

Two other important metabolites formed as a consequence of phosphoinositide hydrolysis are Ins (1,3,4)P_3 and Ins-P_4. EC_{50} values representing the effective concentration of these inositides which acts intracellularly to raise intracellular ionized calcium to 50% of maximal elevation, have been determined for each of these. Unlike Ins (1,4,5)P_3 (EC_{50} = 0.1 uM) whose primary role is releasing Ca^{2+} from the DTS, Ins (1,3,4)P_3 serves to maintain the Ca^{2+} signal. The physiological level of Ins (1,3,5)P_3 formed within the blood platelet is $\geq$ 20 uM (EC_{50} = 20 uM). Ins-P_4 is not involved in Ca^{2+} mobilization (EC_{50} = 20 uM but intracellular concentrations are << 20 uM [96,97].

C) Fate of Ins-P_3 and DAG

The interaction of an agonist with its receptor induces the hydrolysis of PtdIns 4,5-P_2 by phospholipase C, to yield the two intracellular signals DAG and Ins-P_3. Ultimately, it is necessary to resynthesize the lipid precursor so that the cycle may continue. The two products formed are mostly conserved by being fed into a lipid cycle and inositol phosphate cycle, that finally combine to reform PI.

When stimulated, platelets rapidly produce DAG. This DAG is present in membranes only transiently; within a minute of formation it disappears, either returning to inositol phospholipids or becoming further degraded to arachidonic acid (AA) for thromboxane (TXA_2) and prostaglandin synthesis. In addition to the synthesis of inositol lipids via DAG kinase, lipase activities have been described in human platelet particulate fractions. DAG lipase is capable of hydrolyzing DAG to fatty acid and glycerol [98]. Apparently, a sequential removal of stearate at glycerol-sn-1 followed by arachidonate at glycerol-sn-2

occurs via DAG and monoglceride lipase [99], and both stearic and AA are liberated. DAG lipase thus offers one possible route by which AA can be released in activated platelets.

Studies using red blood cells have revealed the presence of an active inositol triphosphatase, which attenuates the second messenger activity of Ins-P_3, by removing phosphate from the 5-position to produce inositol diphosphate [100]. An inositol biphosphatase hydrolyzes inositol diphosphate to inositol 1-phosphate, which is finally converted to free inositol by an inositol 1-phosphatase [100].

D) A Model for Signal Transduction in the Blood Platelet

Under physiological conditions the formation of a haemostatic plug is generally associated with platelet activation normally involving platelet shape change, aggregation and release [1,9,10]. All these reactions are dependent on a primary stimulant such as adenosine diphosphate (ADP) and involve a variety of morphological and biochemical changes leading to the formation of irreversible aggregates in vitro and haemostatic plugs in vivo.

The very earliest and most direct activation of platelets may involve the opening of receptor-operated Ca^{2+} channels leading to early rapid shape change and aggregation with activators like ADP and low concentrations of thrombin. Little is known about the regulation of these receptor-operated channels responsible for calcium influx through the platelet plasma membrane. Some of the possibilities include direct interaction of agonist-occupied receptors with calcium channels, such as for rapid acting ADP; effects mediated by G-protein subunits within the platelet membrane; regulation by soluble second messengers; and phosphorylation of calcium channels [97]. Fluoride, which in the form of

AlF^-_4 stimulates all G-proteins, has been shown to enhance calcium influx into aspirin-treated platelets [101]. This observation suggests the possibility of a direct role for a G-protein in opening Ca^{2+} channels, though it is difficult to exclude effects attributable to the activation of phospholipase C.

The phosphatidylinositol (PI) and endoperoxide pathways are two additional intracellular pathways involved in signal transduction in activated platelets. Their respective enzymes, phospholipase C and phospholipase A_2, have associated with them different G-proteins. These regulator proteins are similar in action to the GTP-binding protein associated with adenylate cyclase. The G-protein is involved in the coupling of the agonist bound receptor to the catalytic subunit of the respective enzyme.

Kaibuchi and others [81,95] have recently shown the occurence of PI metabolism to be the earliest event associated with platelet stimulation. In 1949 Folch isolated phosphoinositides in the brain [102]. Subsequently, they were shown to consist of PtdIns 4-P and PtdIns 4,5-P_2. As previously mentionned, PtdIns 4-P and 4,5-P_2 are produced from PI in situ through sequential phosphorylations of the inositol moiety, by PI and PtdIns 4-P kinases. Initially, Hokin in 1953 believed that PI was the prime target of phospholipase C; but recent evidence seems to suggest that following stimulation of the receptor, PtdIns 4,5-P_2 is immediately degraded to DAG and Ins-P_3 [103].

Occupation of the receptor by an agonist (ADP) results in a conformational change and subsequent activation of the enzyme involved, namely phospholipase C. Rink et al., [104] favor the idea that calcium permeability increases as a direct result of this conformational change produced in the receptor complex. This could be the result of a putative G-protein which mediates the direct effects of ADP and low

thrombin. Agonist-induced activation of phospholipase C is biologically dependent on calcium, but physiologically appears to occur at basal levels of Ca_i^{2+} (< 100 nM). Phospholipase-A_2 (PLA_2), unlike phospholipase C, requires a threshold concentration of calcium to be activated following receptor occupancy [104].

DAG and Ins-P_3 are immediate breakdown products of PtdIns 4,5-P_2. Inositol triphosphate is released as a second messenger from the inner leaflet of the platelet membrane; and possibly acts by interacting with a receptor on the surface of the dense tubular system (DTS). The DTS is an intracellular storage pool for calcium. Ins-P_3 binds, and results in the mobilization of calcium from the DTS into the cytoplasm. The elevated cytoplasmic calcium binds to a cytosolic protein, calmodulin; which, subsequently leads to the activation of myosin light chain kinase and resultant shape change (morphological and biochemical changes) and early platelet recruitment [81]. PtdIns 4,5-P_2 chelates calcium, which when cleaved by phospholipase C liberates additional calcium into the cytosol.

Diacylglycerol, the second product of PtdIns 4,5-P_2, serves as an activator of protein kinase C. This enzyme is associated with the phosphorylation of a 47,000 MW protein in the cytosol, which may be involved in platelet secretion [97].

Platelet activation is both time and concentration dependent. With 10 uM ADP, enough Ins-P_3 is generated from the hydrolysis of PtdIns 4,5-P_2 to induce platelet shape change (SC) and partial aggregation (PA); however, the system has not accumulated threshold amounts of DAG required for secretion. The Ins-P_3 formed mobilizes sufficient calcium (0.3 uM) to activate the threshold-dependent phospholipase-A_2 (PLA_2). Once PLA_2 is stimulated, arachidonic acid (AA) is released from membrane phospholipid. AA is rapidly taken up by the DTS where it is

cyclicized and oxygenated to endoperoxides PGG_2/PGH_2. These proaggregatory agents are then acted upon by TXA_2 synthetase, which gives rise to TXA_2 [105]. TXA_2, like Ins-P_3 has the ability to modulate intracellular calcium, thus further driving shape change and aggregate formation [97].

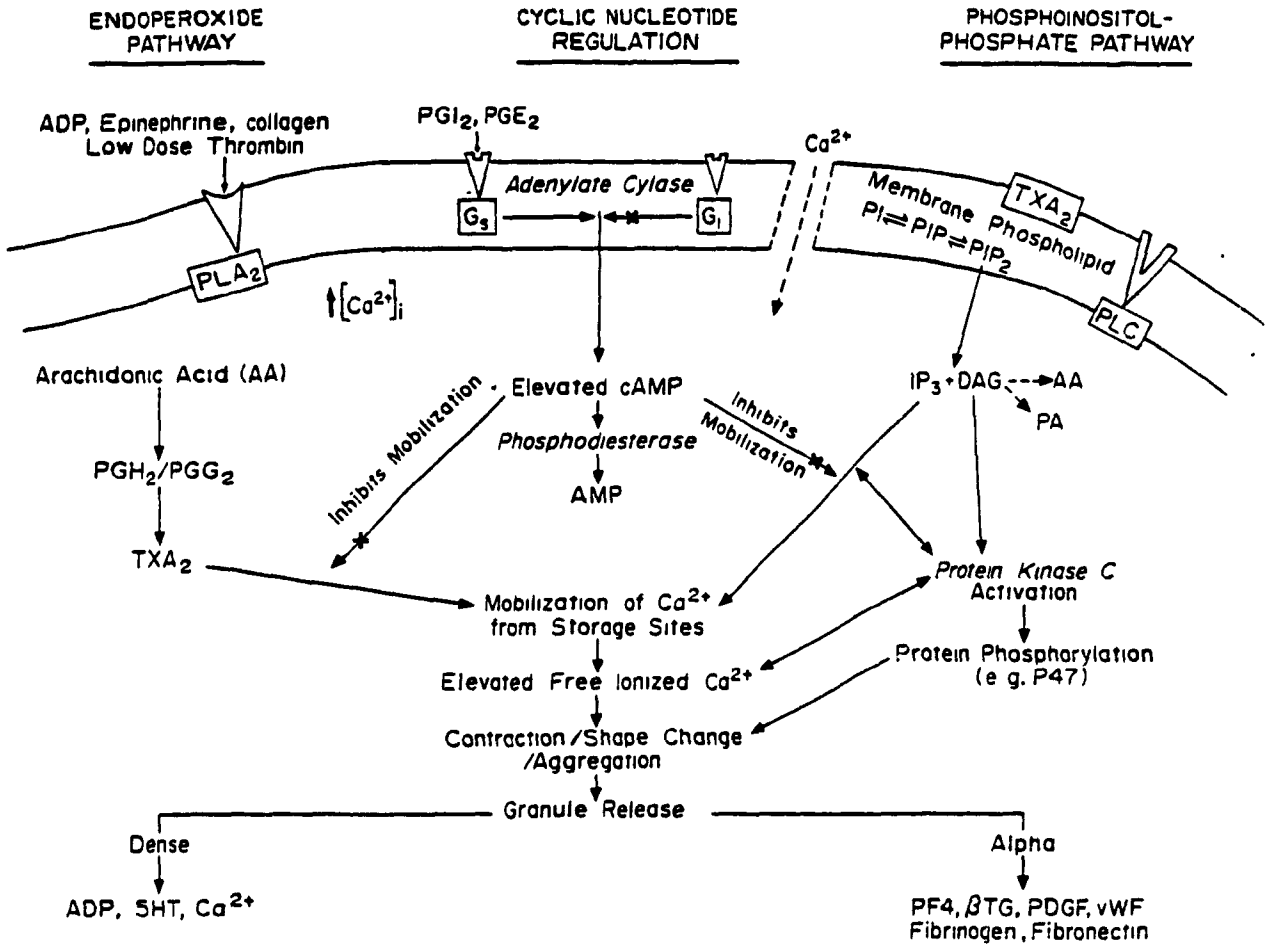
The TXA_2 generated primarily exerts positive feedback control on phospholipase C and increases its activation; thus, producing sufficient DAG to activate protein kinase C and promote secretion. The threshold level of intracellular calcium for secretion is ~ 0.6 uM [105] (i.e. Ins-P_3 and DAG normally act in concert under maximal activation). However, protein kinase C appears to be activated at low basal levels of intracellular ionized calcium i.e., it may undergo enhanced sensitivity to low Ca^{2+} and be otherwise independent of any elevations in calcium [101] (see Fig. 8).

IX. Platelet Inhibition and its Role in Signal Transduction

A) Overview

Platelet inhibition and negative feedback control of platelet activation appear to be most widely mediated by platelet intracellular cAMP [81], which generally causes recovery from or opposes the action of second messengers associated with platelet activation such as calcium and others derived from inositol lipid hydrolysis, such as Ins-P_3 and DAG [81,106]. Cyclic-AMP can also suppress a number of other intracellular components distal to the elevation of intracellular calcium or the formation of DAG [107]. There is increasing evidence that regulation of platelet activation involves changes in both concentrations and types of regulatory molecules with different threshold concentrations of intracellular calcium for example, driving

Figure 8: Signal transduction in platelet activation and inhibition: The role of intracellular calcium (Ca_i^{2+}) and diacylglycerol (DAG).



platelet activation to different extents [107].

The best known and most potent physiologic inhibitors of platelet function are the prostaglandins I_2 (prostacyclin) and E_1 [44]. These receptor-active agents stimulate adenylate cyclase activity resulting in a substantial rise in platelet intracellular cAMP associated with platelet inhibition [108]. However, it has also been suggested that PGE_1 can cause inhibition at concentrations too low to yield measurable increases in cAMP [109].

B) History of Prostacyclin (PGI_2)

Moncada and Vane found a new substance in 1976 from the group of arachidonic acid metabolites which was first named prostaglandin X or PGX [110-112]. After isolation, structural elucidation, and chemical synthesis, the compound was renamed prostacyclin (PGI_2). After the isolation of PGI_2 and detection of its unique properties [110-113], numerous studies have been performed that were designed to take advantage of its desirable pharmacological properties for clinical medicine. Indeed, most of the available data suggest beneficial effects of exogenous PGI_2 in situations which are thought to be connected with a reduced endogenous availability or enhanced requirement of PGI_2. Examples are obstructive vessel diseases [114], hemolytic uraemic syndrome [115] or pulmonary bypass [116].

The predominant biological effects of PGI_2 are vasodilatory and the ability to prevent the clumping of platelets or to break up existing aggregates. Indeed, PGI_2 is the most potent endogenous vasodilator and inhibitor of platelet aggregation. It also inhibits the transformation of GpIIb-IIIa complexes in vitro into functional fibrinogen receptors, and thus inhibits platelet fibrinogen interactions. PGI_2 also enhances fibrinolytic activity (induces a protease plasminogen activator) [51].

The vasodilatory action of PGI_2 can be utilized for the treatment of raised blood pressure. On the other hand the inhibiting action on platelet aggregation is suitable for prophylactic and acute treatment of thrombosis and disturbances of the peripheral circulation. It has not proved useful to-date for preventing or managing thrombo-embolic diseases associated with TIA or stroke [117]. Some of the apparent problems arise from 1) the limits on PGI_2 that can be used without the complicating side effects of vasodilation and decreasing blood pressure [118,119], 2) platelet refractoriness to PGI_2 seen to develop in some of these clinical disorders [120,121] and 3) a reduced free concentration of PGI_2 due to the absorption by blood cells and/or enhanced metabolic breakdown seen for example after ischemic stroke [122].

Platelet activation is involved in the pathogenesis of obstructive coronary disease. For this reason the endogenous prostaglandin, PGI_2, has been proposed as a promising agent to protect the ischemic myocardium. It has been shown that the inhibitory effect of PGI_2 on in vitro aggregation is less marked in patients with acute myocardial infarction than in normal volunteers [123-125]. To explain this attenuated response to PGI_2, Buttrick et al [123] postulated that platelets from infarcting patients were qualitatively different from those of normal subjects; this may be due to elevated catecholamine contents associated with infarction.

The natural candidate for study is PGI_2, the most potent endogenous vasodilator and inhibitor of platelet aggregation known to date [126]. However, there are a number of inherent problems. PGI_2 is a chemically unstable prostaglandin compound, its half-life in blood or physiologic solutions being in the range of a few minutes. PGI_2 is a natural product and, therefore, as seen with other members of the prostaglandin family, undergoes metabolic

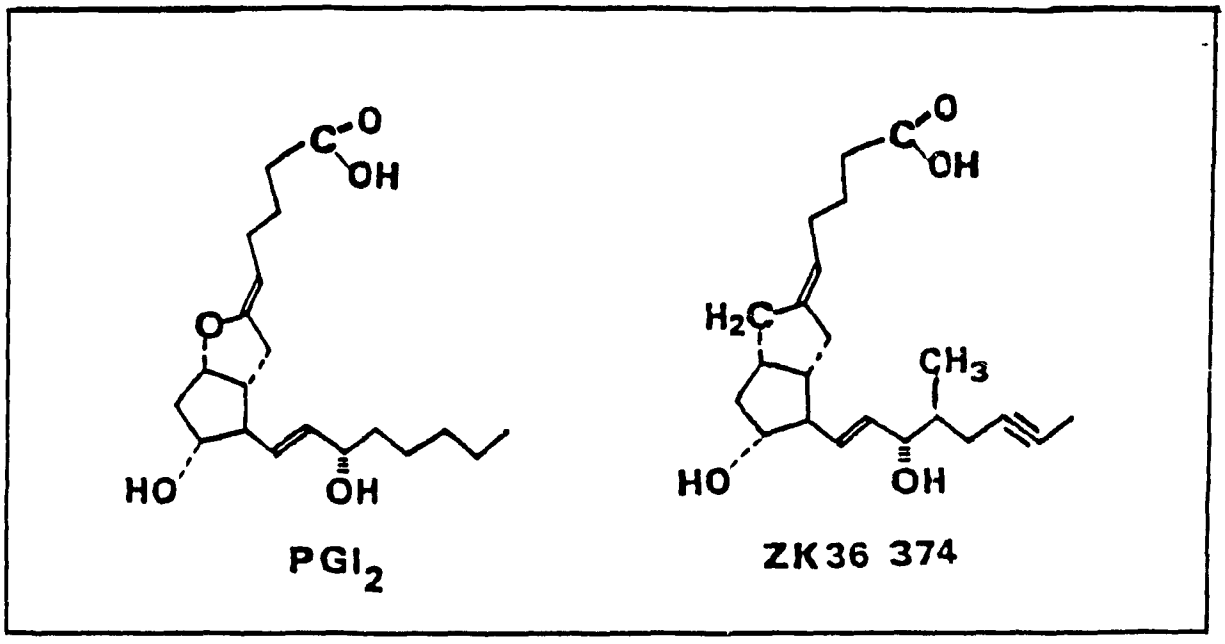
transformations in situ e.g., into several PGF-like compounds [127,128]. PGI_2 is a powerful vasodilator, and therefore lowers blood pressure in vivo. Although this action may be desired for some conditions, it is an unwanted side-effect in others; particularly, when only its antiplatelet activities are wanted. Complications using PGI_2 can be by-passed using Iloprost (ZK 36374), its chemically stable carbacyclin derivative. ZK shows a similar profile of action as PGI_2 for inhibition of platelet functions, with 2-5 times greater potency observed for some in vitro tests [113]. ZK has a methyl group at C-16 and a triple bond in the 18-19 position (see Figure 9).

Iloprost has been found to exert antiplatelet and tissue-protective actions (cardioprotective) in laboratory animals at doses that did not decrease systematic arterial blood pressure and in this respect it was clearly superior to PGI_2 in vivo [129]. A dissociation between the antiplatelet and blood-pressure lowering activities of Iloprost was also suggested recently following studies in healthy volunteers [130-132]; however at high doses both compounds exert a direct vasodilatory action [133]. Following the original observation by Ogletree et al., numerous studies have confirmed this cardioprotective potential of ZK in acute myocardial ischemia [129,134-137]. It has been proposed as an approach to the therapy of stable and unstable angina pectoris because of its powerful vasodilatory and antiplatelet effects [129,138].

X. Factors Affecting Platelet Function in Whole Blood

Most in vitro studies of the effects of physiologic activators and of inhibitors such as prostacyclin (PGI_2) on platelets have been performed using platelet-rich plasma (PRP) prepared by centrifugation of anti-coagulated whole

Figure 9: The chemical structure of ZK 36 374 as compared to PGI_2. (Schror, K., Darius, H., Matzky, R., Ohlendorf, R. Arch. Pharmacol. 316: 252-255, 1981).



blood. The use of PRP for platelet aggregation studies has at least three main disadvantages: 1) unstable in vivo metabolites and their physiological effects on platelets may decay over 1-2 hours post-bleeding time normally used in preparing and studying PRP; 2) because platelets are heterogenous in terms of size, density and metabolic activities [24,139], certain populations of platelets may not be recovered by centrifugation. Indeed, mean % recovery is ~ 67% [140], though the size distribution of residual platelets has not been compared to that of the platelets actually isolated in the PRP; any differential sedimentation of platelets would however favor the loss of larger, denser platelets [139]. Therefore, the results of aggregation studies on PRP may not represent platelet function in whole blood; and 3) in PRP, platelet aggregation occurs in the absence of red and white blood cells, thus pre-empting the detection of any possible effects of these cells on platelet function. Platelet behaviour in whole blood can be very different from that in PRP. Red blood cells in flow are believed [141-143] to enhance platelet aggregation by releasing adenosine 5'-adenosine diphosphate (ADP). On the other hand others have reported that aggregation in whole blood needs larger amounts of ADP than in PRP, probably because of the presence of red and white blood cells, whose plasma membrane is rich in ADPase and 5-nucleosidase activity [144-146]. In addition, white blood cells may reduce platelet aggregation by generating PGI_2 [147,148]. A significant negative correlation was found between the count of WBC's (polymorphonuclear [PMN] leukocyte) and measured whole blood platelet aggregation [149]. These findings agree with an earlier report by Harrison and colleagues [150], which showed a progressive inhibition of platelet aggregation with the artificial enrichment of PRP with WBC, using the Born aggregometer. Therefore, there is a need to re-evaluate some of the pharmacological agents known to

modify platelet function in PRP.

Adenosine is a potentially important antithrombotic substance. This nucleoside is a potent vasodilator produced in increased amounts in conditions of tissue ischaemia [151]. It is also a potent antiaggregating substance working through stimulation of platelet adenylate cyclase [152]. Although it is rapidly taken up and metabolized by the red blood cell, it may still affect assays conducted in whole blood.

Even the stability of PGI_2 in whole blood and PRP differ. By measuring the disappearance rate of labelled PGI_2 during a 37°C incubation, one finds using a quantitative chromatographic method that the half-life of PGI_2 in whole blood is 6.3 ± 0.8 minutes significantly shorter than the 10.7 ± 2.3 minutes in PRP [153].

Spontaneous platelet aggregation (SPA) has been reported to affect platelet studies in whole blood [154]. It has been shown that ADP can leak out of the cell with time ($\geq$ 30 minutes) and affect platelet sensitivity. To minimize any apparent red cell-induced spontaneous platelet aggregation in whole blood, controls must be adopted with respect to stirring time (see Methods chapter 2). SPA is occasionally observed in whole blood from patients with myocardial infarction or cerebral ischaemia [155-157] as well as in asymptomatic individuals [158]. Although the precise mechanism of this phenomenon is unknown, enhanced sensitivity of platelets to ADP has been suggested [156]. On occasion individual donors have shown marked increases in disco-echinocytes in unactivated samples of whole blood, due to spontaneous activation.

XI. Methodologies for Studying Platelet Function in Whole Blood.

The Wellcome aggregometer is an electrical instrument developed by Cardinal and Flower [159] for studying platelet aggregation. It differs from the turbidometric aggregometer in that it can be used to measure platelet function in whole blood, without first separating platelets from erythrocytes and white cells. Instead of measuring light transmittance (%T), platelet aggregation is measured directly, as a change in resistance between two electrodes immersed in whole blood. A pair of electrodes are placed in a blood sample and the increase in impedance is recorded as aggregating platelets accumulate on them. This change in resistance is related to the mass of the platelet aggregate on the electrode tip, and in turn measures macroaggregation related to the concentration of the aggregating agent added [160].

Another method for studying platelet function in whole blood, involves the use of an electronic cell counter. In order to count platelets in whole blood samples, fixed PRP is prepared by desk-top centrifuge spun at 150 x g for 2 minutes Ten microliters (ul) of fixed PRP is dispensed into 10 mls of isotonic diluent (Hematall) and the platelet particle count was determined with a resistive particle counter. Percent microaggregation in whole blood is determined from the percent decrease in the particle count.

Similarly platelet function may be quantitated by microscopic measurements of platelet shape and aggregate formation. Using fixed PRP prepared from whole blood, phase contrast microscopy is used to study platelet morphology i.e., the percent of echinocytes (shape-changed platelets) distinct from unactivated smooth-surfaced discocytes. Percent aggregate formation is determined by the percent decrease in the single platelet count.

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Chapter II

Differential Inhibition of the Platelet Activation Sequence: Shape Change, Micro- and Macro-Aggregation, by a Stable Prostacyclin Analogue (Iloprost)

Key words

Differential activation and inhibition - Shape change-Microaggregation - Macroaggregation - Iloprost (ZK 36374)

Summary

The relative sensitivities of adenosine diphosphate (ADP)-induced activation, and of prostaglandin-mediated inhibition, were determined for rates of platelet shape change (SC [V_S]), early platelet recruitment measured by electronic platelet counting (PA [PA_3]), and turbidometrically-measured aggregation (TA [V_a]). Studies were performed in stirred citrated platelet-rich plasma from 9 healthy human donors. The $[ADP]_{1/2}$, ([ADP] giving half maximal rate) was determined for the sequence of activation steps: unactivated platelets -> SC -> PA -> TA. Distinct ADP sensitivities were obtained from log dose-response studies, with a relative dose dependency for rates of change in the order of $[ADP]_{1/2}$ TA > $[ADP]_{1/2}$ PA > $[ADP]_{1/2}$ SC of ~4:3:1. Differential inhibition of the above activation scheme was evaluated from log dose-response curves for Iloprost (ZK 36374), a stable carbacyclin analogue of prostacyclin (PGI_2), with greater potency than PGI_2 for the same platelet receptors. IC_{50} values corresponding to Iloprost concentrations causing 50% inhibition of rates of TA (V_a), PA (PA_3) and SC (V_S) were found in the relative ratios of 1:~3:~5, when measured at a common ADP concentration for all three parameters, or 1:~2:~3 when determined at respective $[ADP]_{1/2}$ values for each parameter. Thus about 3-5 times more Iloprost is required to respectively inhibit the rates of shape change (V_S) and early platelet recruitment (PA_3), than that needed to inhibit the rate of turbidometrically-measured aggregation (V_a).

Introduction

Under physiological conditions, the formation of a haemostatic plug is generally associated with platelet activation normally involving platelet shape change, aggregation and release (1). All these reactions, dependent on a primary stimulant such as adenosine diphosphate (ADP), involve a variety of morphological and biochemical changes leading to the formation of irreversible aggregates in vitro and haemostatic plugs in vivo. Platelet inhibition and feedback control appear to be most widely mediated by platelet intracellular cyclic AMP (2), which generally causes recovery from or opposes the action of second messengers associated with platelet activation such as calcium and others derived from inositol lipid hydrolysis, such as inositol triphosphate and diacylglycerol (2,3). Cyclic AMP can also suppress a number of other intracellular components distal to the elevation of intracellular calcium or the formation of diacylglycerol (4). Prostacyclin is the most potent physiological inhibitor serving these roles (5). There is increasing evidence that regulation of platelet activation involves changes in both concentrations and types of regulatory molecules with different threshold concentrations of intracellular calcium for example, driving platelet activation to different extents (2-4).

Under maximal stimulation, the different phases of platelet activation appear to be part of a continuum. However, previous studies using ADP as activator have shown the onset of shape change (SC) to be distinct from subsequent early platelet recruitment (PA). Singlets appear fully recruited before the formation of large aggregates ($\geq$10 per aggregate) or detection by aggregometry (TA), suggesting the sequence: SC -> PA -> TA (6-9). This

allows an expansion of Holmsen's classic scheme for platelet activation (10) based on studies focused on turbidometrically-measured shape change (SC), primary aggregation (TA-1), secondary aggregation (TA-2) and associated release reaction, with each step requiring increasing stimulus concentrations (10): namely, SC -> PA -> TA-1 -> release and TA-2.

Little has been reported on the dynamics and sensitivity for inhibition of the overall sequence of activation events, as seen from reviews on both prostacyclin (5) or cAMP-mediated (10) inhibition. Holmsen and Steen (11) have reported no distinct difference in the inhibitory action of PGE_1 and adenosine on platelet shape change and macroaggregation, in response to a common high concentration of ADP (4.5 or 14 uM). However, others have shown that lower concentrations of inhibitors [PGE_1 (12), anagrelide (13), and halofenate (14)] are required to block TA-2 and release, than are needed to inhibit shape change, when generally evaluated at one common high ADP concentration (10 uM). More recently, inhibition of shape change was reported to require ~10x more PGD_2 than that required for comparable inhibition of TA-2 and release, measured for concentrations of platelet-activating-factor causing ~70% of maximal platelet responses (4). Finally, studies of platelet aggregation measured by singlet counting have indicated that the early aggregation process (PA), largely undetected by turbidometric measurements (TA), has distinct properties from TA as evaluated for PGE_1, anagrelide and EDTA as inhibitors and ADP as activator (6). We therefore sought to establish the sensitivity for inhibition of the overall sequence of platelet activation over a range of ADP concentrations using a potent prostaglandin inhibitor.

The most potent endogenous vasodilator and inhibitor of platelet aggregation is prostacyclin (PGI_2) (5). However, complications caused by PGI_2's instability in aqueous medium

can be bypassed by using ZK 36374 (Iloprost, henceforth referred to as ZK), its chemically stable carbacyclin derivative (15,17). ZK shows a similar pharmacological profile as PGI_2 for inhibition of platelet functions (15), with 2-10 times greater potency observed for some in vitro tests (16,17).

Using electronic particle counting (8), rather than previously reported singlet platelet measurements obtained with a haemocytometer (6,8), in parallel with turbidometric measurements (6), we now report the differential ADP sensitivity ($[ADP]_{1/2}$) for the sequence of unactivated platelets -> SC -> PA -> TA. We then evaluate the relative amounts of ZK needed to inhibit this sequence using a common [ADP] for all three steps in the activation sequence or a [ADP] corresponding to respective $[ADP]_{1/2}$ values determined for each activation step.

Materials and Methods

Preparation of Platelet-Rich Plasma (PRP)

Normal donors were chosen from healthy men and women between the ages of 20 and 50 years. Ingestion of aspirin or any other non-steroidal anti-inflammatory drugs was prohibited at least 2 weeks prior to donation. Blood was drawn by venipuncture into 3.8% citrate (one volume to 9 volumes blood), and PRP was prepared at 37°C and pH = 7.4 ± 0.1 as previously described (18).

Dose Response Studies of Shape Change and Aggregation Measured Turbidometrically

ADP (1-10 ul) was quickly injected using a Hamilton syringe (Hamilton Co., Reno, Nev.) into 0.4 ml platelet

suspension (PRP) in a cuvette (6.9 mm x 45 mm) with stir bars (6 mm x 1 mm) spun at 1000 rpm, 37°C. Changes in light transmission were monitored using an aggregometer (Payton dual channel aggregation module), with a rapid chart speed as previously described (13). The slope of the initial decrease in light transmission, V_s, and that corresponding to the maximal rate of increase in light transmission, V_a, were respectively measured as rates of shape change and aggregation, as previously described (13) (Fig. 2). Maximal values of V_s and V_a were obtained for [ADP] = 10 uM. The [ADP] required to induce one-half maximal rate of SC (V_s) and TA (V_a), $[ADP]_{1/2}$, was readily determined from a log dose-response plot of $V_s/(V_s)max$ or $V_a/(V_a)max$ versus [ADP] in the range of 0.1-10.0 uM (Fig. 1).

Aggregation from Platelet Counting (PA)

Platelet microaggregation (PA) was estimated from the percent decrease in the particle count determined with an electronic (resistive) particle counter as previously described (8); in one experiment, PA was also determined from singlet counting using a haemocytometer (6,8). A 0.1 ml volume of PRP was placed in a cuvette and stirred at 1000 rpm. At the indicated time, 0.5 ml of 0.8% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) was added directly to the cuvette. Ten microlitres (ul) of fixed platelet suspension was dispensed into 10 ml of Hematall isotonic diluent (Fisher Scientific Co., Pittsburgh, Pa), and the platelet particle count was determined with a resistive particle counter (Elzone, Particle Data Inc., Elmhurst, Ill.) (8). The percent platelet aggregation, PA_t, was calculated from the following relation:

$$\%PA_t = [1-N_t/N_o] \times 100\%$$

Fig. 1 Log dose-response curves for ADP-induced rates of shape change (V_S), early platelet recruitment (PA_3) and turbidometrically-measured aggregation (V_a). Typical curves are shown for relative changes in V_S, PA_3 and V_a for a single donor as described in the methods. $[ADP]_{1/2}$ values are listed in Table 1.

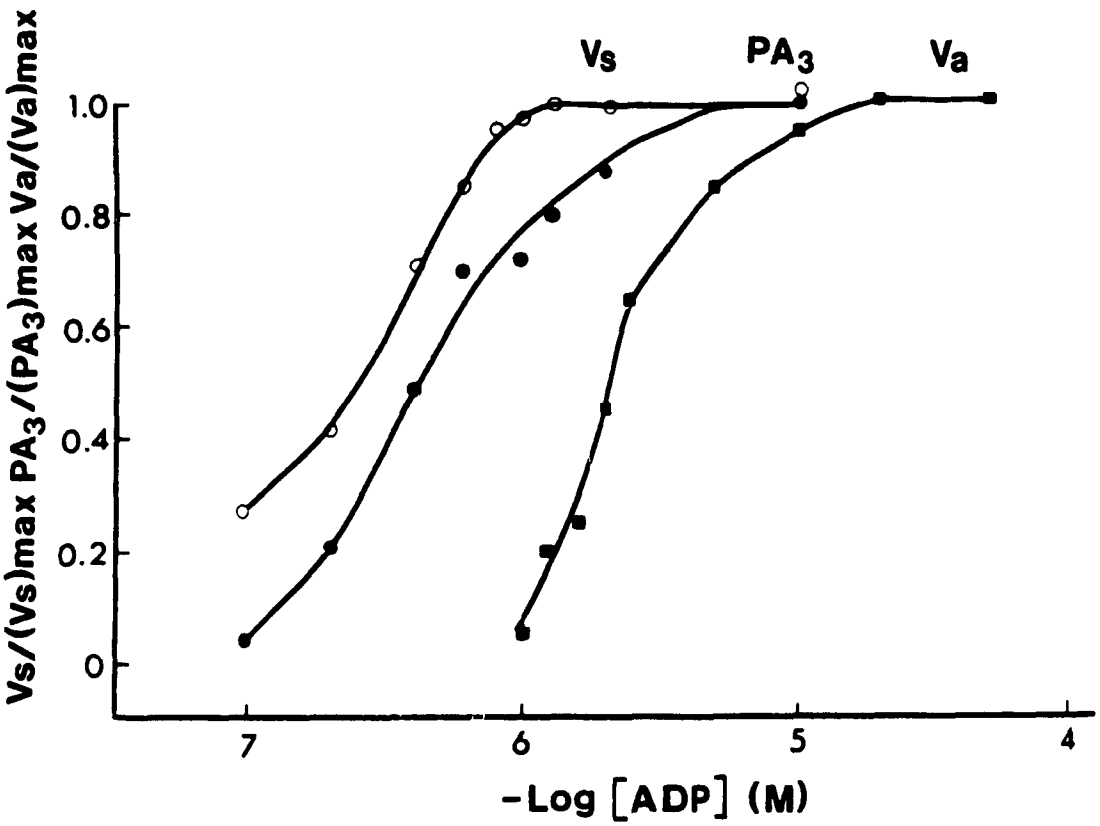
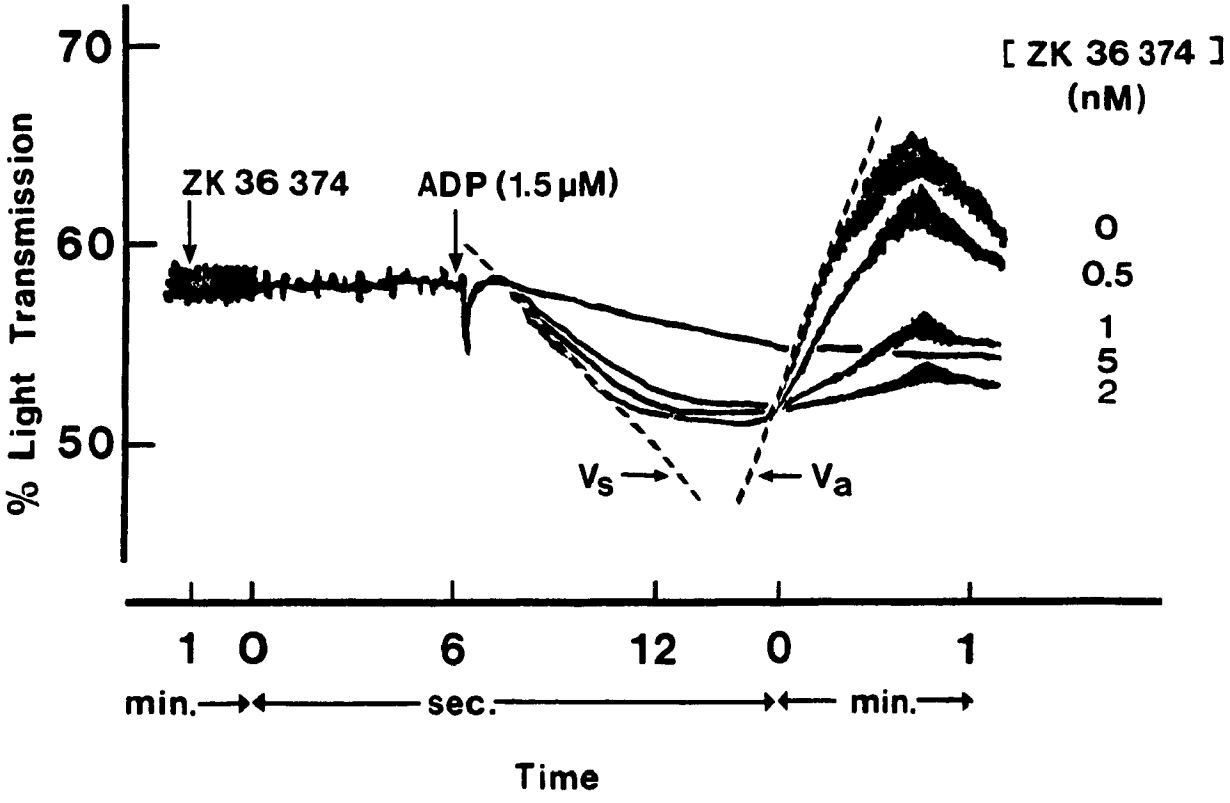


Fig. 2 Typical turbidometry tracings for a single donor showing increasing inhibition of ADP-induced rates of shape change (V_s) and of aggregation (V_a), with increasing ZK 36374. PRP was stirred with increasing ZK for 1 minute preceding addition of ADP, with parameters for analysis as described in Methods.



where N_t, N_o are respectively, the platelet singlet (haemocytometer) or particle (electronic) counts at times t and zero. PA was measured at 3 seconds corresponding to the initial linear rate for early platelet recruitment (6,8). $[ADP]_{1/2}$ values were routinely obtained from PA at 3 seconds measured for [ADP] from 0.2 to 10 uM, with the same graphical analysis as described above for V_s and V_a (Fig. 1). Unless otherwise indicated, PA measurements are based on electronic counting.

Inhibitor Studies

ZK (Iloprost, ZK 36374) was incubated with PRP ($\leq$ 5% v/v PRP; final concentration ranged from 0.5 to 10 nM) at 37°C, one minute prior to the addition of ADP. ZK, with one minute incubation in PRP or in washed platelets was 2.5 to 10 times more potent than PGI_2 in elevating basal cAMP levels in human platelets (17,19).

ZK concentrations causing 50% inhibition of PA_3, V_s or V_a (IC_{50} values), were derived from a plot of % inhibition calculated from $PA_3/(PA_3)max$, $V_s/(V_s)max$ or $V_a/(V_a)max$ versus [ZK 36374] determined for a common [ADP] (~1.5 uM) (Fig. 3) or at the $[ADP]_{1/2}$ for the specific parameter being tested.

Dose-Response Studies of Shape Change Measured Microscopically

Although it was previously shown by direct microscopy that the initial rate of decrease in light transmission of PRP in the first 5 seconds following ADP addition represents changes in platelet shape (20,21), independent of any parallel microscopic aggregation (21), we re-confirmed these observations to ensure no artifactual effects of ZK on these measurements. Thus, ADP added to PRP at 1000 rpm, 37°C was

stirred for ≤ 1 second to ensure complete mixing and minimal aggregation; incubated for 1-10 seconds, and all morphological changes arrested with 0.8% glutaraldehyde (0.5 ml/0.1 ml PRP) (20). The choice of 5 seconds post ADP addition was re-confirmed as appropriate for measuring the initial linear rate of platelet shape change (20). For inhibitor studies, PRP was pre-incubated with ZK for one minute as above.

Materials

Trisodium citrate (J.T. Baker Chemical Co., Phillipsburg, N.J.), and ADP (Sigma Chemical Co., St. Louis, Mo.) were prepared in Ca^{+2}/Mg^{+2} free Tyrodes buffer as previously described and kept frozen (-20°C) as a stock solution (18). ZK (Iloprost; ZK 36374) was a generous gift from Dr. Th. Krais, Schering AG, Berlin, West Germany. It was prepared as a frozen (-20°C) 10uM stock solution in saline (0.9% NaCl and 0.125% sodium bicarbonate, pH = 8.2). Glutaraldehyde E. M. Grade (8% stock in sealed ampoules [Polysciences Inc., Warrington, Pa.]) was prepared in modified Tyrodes buffer. Final vehicule dilutions both for ADP and ZK were ≤ 5% v/v in PRP.

Results

Typical log dose-response curves for rates of ADP-induced shape change (V_S), aggregation measured by electronic particle counting (PA_3), and aggregometry (V_a), are shown for a single donor in Fig. 1. Table 1 summarizes the relative ADP sensitivities for these three processes determined from such curves for 7 donors. These relative sensitivities appear to be independent of donor variations. The relative dose dependency for platelet activation by ADP

is in the order TA > PA > SC; approximately in the ratio of 1:3:4. It appears that progressively larger concentrations of ADP are required to drive platelet shape change, early recruitment, and build-up of larger aggregates.

Turbidometrically measured rates of shape change (V_s) and aggregation (V_a) were increasingly inhibited with a progressive increase in ZK (Iloprost) added to PRP for one minute preceding ADP addition, as shown in Fig. 2 for 1.5 uM ADP. Similar observations were made in parallel studies for ZK-inhibition of the rate of early platelet recruitment (PA_3). Log dose-response curves were obtained for ZK-mediated inhibition of V_s, PA_3 and V_a conducted in parallel at a common ADP concentration sufficient to drive all these uninhibited processes by $\geq$50% of their maximal rates. A typical set of such curves for [ADP] = 1.5 uM is shown in Fig. 3 for 1 donor; ZK concentrations causing 50% inhibition (IC_{50}) of V_s, PA_3 and V_a are derived from such curves. Results for such studies obtained at a common [ADP] show relative IC_{50} values for V_s, PA_3 and V_a to be ~5:~2:1 respectively (Table 2). This suggests that ~5 and ~2 times more inhibitor is required to comparably inhibit V_s and PA_3 than V_a respectively, when measured at one common ADP concentration (~1.5 uM).

As the IC_{50} value for any given parameter will vary with the particular [ADP] tested, and since our three parameters are distinct with respect to ADP sensitivity, we also determined IC_{50} values for ZK in parallel experiments at the $[ADP]_{1/2}$ value for each parameter. These IC_{50} values were determined in parallel for each donor, with relative values pooled for 9 donors as shown in Table 2. We again observed a differential sensitivity to ZK as inhibitor, with ~3 and ~2 times more ZK needed to inhibit rates of shape change (V_s) and microaggregation (PA_3) than that required to inhibit macroaggregation (V_a).

Table 1 Relative ADP sensitivities for rates of shape change and aggregation

Parameter	$[ADP]_{1/2}$[1] uM	Relative[2] $[ADP]_{1/2}$
Rates of shape change:		
Turbidometry (V_s)	0.6 ± 0.3	1.0
Rates of aggregation:		
Particle counting (PA_3)	1.4 ± 0.3	2.7 ± 1.5
Turbidometry (V_a)	1.8 ± 0.4	3.5 ± 1.5

[1]Mean ± SD for mean values obtained for 7 donors (9 experiments)

[2]Relative values obtained by comparing $[ADP]_{1/2}$ for three parameters for each donor, normalized to 1.0 for V_s, and then averaging for all the donors. Relative values for V_a:PA_3 with PA_3 normalized to 1.0 was 1.3 ± 0.03. Tests for significance of differences were made using a Student's paired t-test determined for paired values for each donor, then averaged. Paired analysis gave $p \leq 0.005$ for comparisons of each pair from the 3 parameters

Fig. 3 Relative-IC_{50} values for ZK 36374-inhibition of shape change and aggregation induced by a fixed ADP concentration (1.5 uM) for an individual donor. Plots are shown for analysis of inhibition of rates of turbidometrically-determined shape change (V_s) (-▲-), of particle-counting-derived early aggregate formation (PA_3) (-O-) and of turbidometrically-measured aggregation (V_a) (--●--). IC_{50} values were respectively 2.8, 1.4, and 0.55 nM for V_s, PA_3 and V_a in this male donor (N_o = 298,575 platelets ul^{-1})

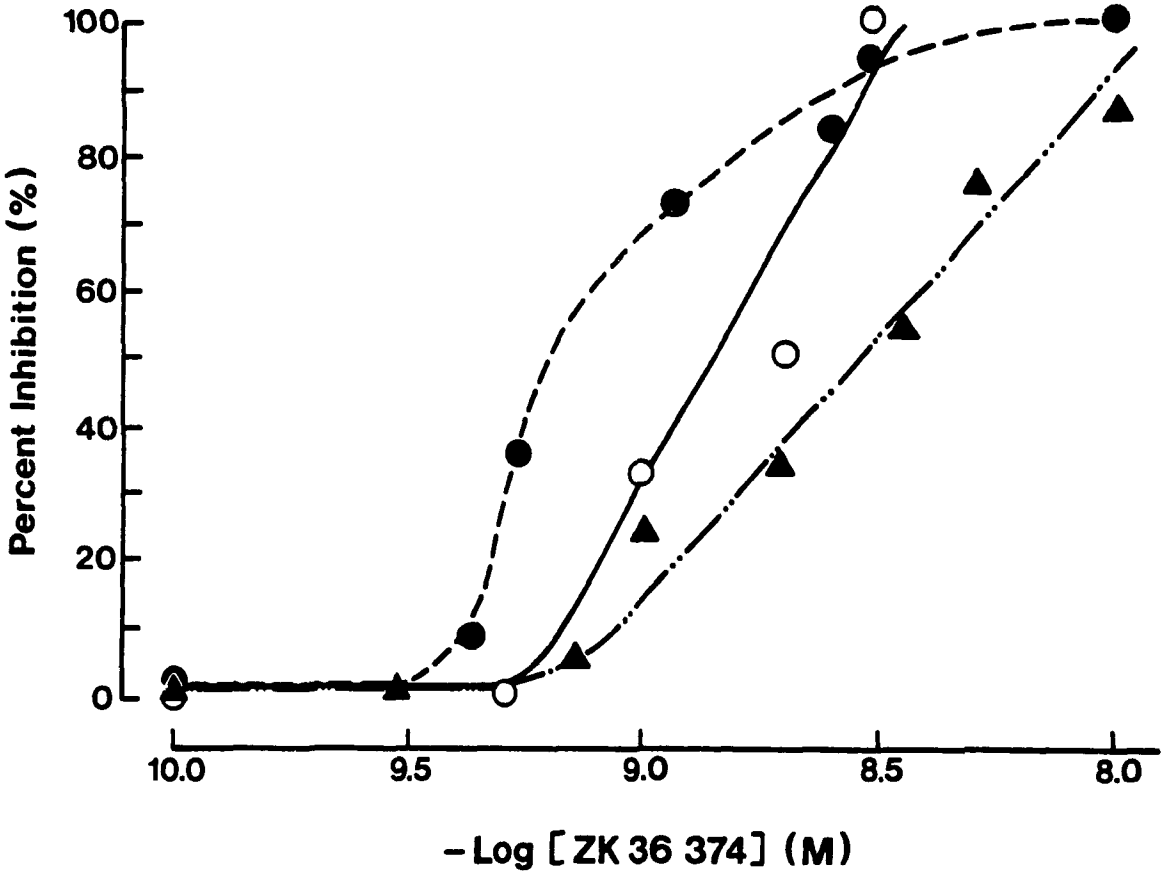


Table 2 Relative ZK sensitivities (IC_{50}'s) for inhibition of rates of platelet shape change and aggregation at a common [ADP] or at the respective $[ADP]_{1/2}$

Parameter	Common [ADP][1]		Respective $[ADP]_{1/2}$[2]	
	ADP Tested	Relative IC_{50}[3]	ADP Tested	Relative IC_{50}[3,4]
Rates of shape Change (V_s)	1.7 ± 0.3	4.5 ± 1.3	0.7 ± 0.3	2.7 ± 0.9
Rates of aggregation (PA_3)	1.5	1.8 ± 0.4	1.5 ± 0.1	1.6 ± 0.4
(V_a)	1.7 ± 0.3	1.0	1.7 ± 0.4	1.0

[1]Data for 5 donors on 6 occasions expressed as Mean ± SD

[2]Data for 9 donors on 10 occasions expressed as Mean ± SD

[3]The IC_{50} values for all three parameters were compared relative to Va normalized to 1.0 for each donor, and then averaged for all the donors. Significance of differences (paired t-test) for each parameter pair was $p \leq 0.005$

[4]Relative values for $PA_3:V_s$ with PA_3 normalized to 1.0 for each of the donors and pooled was 1.8 ± 0.8

In light of the large differences between the IC_{50} values for shape change and aggregation, we next tested the possibility that turbidometrically-measured rates of shape change (V_s) determined as a function of ZK-inhibition might have artifacts associated with platelet volume or refractive index changes (21). Log dose-response curves for ZK-inhibition of V_s induced by 1.5 uM ADP determined for three donors by direct microscopy as described in Methods were essentially identical to those obtained by turbidometry (example in Fig. 3). Thus, IC_{50} values for V_s obtained from such curves derived in parallel experiments for each of three donors were 3.0 $\pm$ 0.9 nM and 3.2 $\pm$ 0.6 nM respectively for microscopic and turbidometric determinations (range 2.3-4.0 and 2.5-3.6 nM respectively).

Since we observed distinct relative values in IC_{50} for the two procedures shown in Table 2, we investigated the relationship between IC_{50} values and the particular [ADP] tested. A plot of all IC_{50} values measured for 12 different donors and the corresponding [ADP] tested showed a linear correlation for rates of shape change (V_s), microaggregation (PA_3) and macroaggregation (V_a), with relative slopes of 5.1:2.7:1 (see Fig. 4). However, this relationship can be considered linear only over the range of [ADP] actually tested, particularly in the case of V_s which has a significant non-zero Y-intercept. Thus, measuring relative IC_{50} ratios at the low and high ADP concentrations in Fig. 4 gives relative values of ~5-6:2:1 for V_s:PA_3:V_a, comparable to results shown in Table 2 for 1.5 uM ADP. We next measured IC_{50} for a given donor over a range of [ADP] covering an ~2-3-fold deviation ($\pm$) from the $[ADP]_{1/2}$ value for any given parameter (Table 3), and found a relative constancy in IC_{50} expressed per uM ADP for any given parameter at each [ADP] evaluated, as shown in Table 3. Indeed when the IC_{50} values in Table 2 determined at respective $[ADP]_{1/2}$ values for V_s, PA_3 and TA are also

Fig. 4 The linear relationship between IC_{50} and ADP tested for rates of shape change (V_s), microaggregation (PA_3) and macroaggregation (V_a) is shown for 12 donors on several occasions. Linear regression was used to obtain a best fit for the data. Correlation coefficients (r) for V_s, PA_3 and V_a are 0.8, 0.8 and 0.6, respectively; slopes are 2.0, 1.0 and 0.4 respectively, with corresponding Y-intercepts of 1.0, 0.1 and 0.3

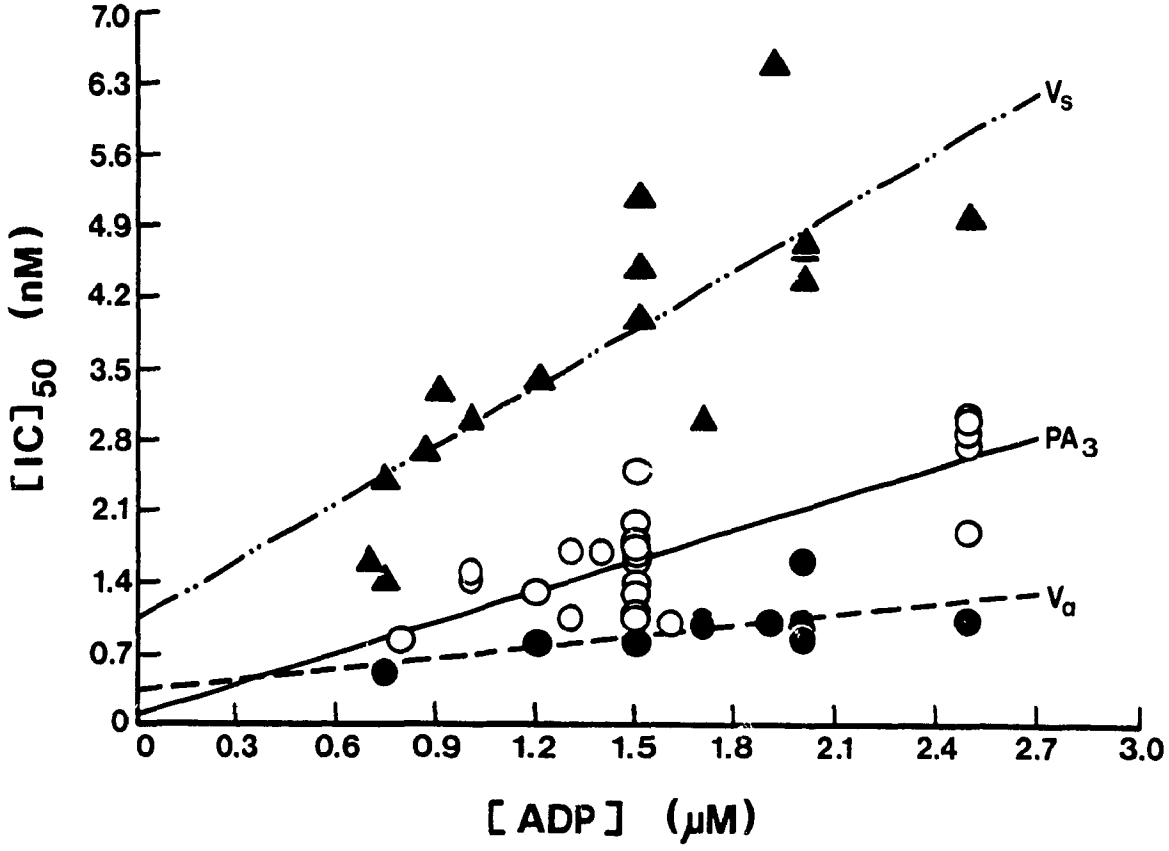


Table 3 ZK-IC_{50} dependence on [ADP] tested for any given donor for a range of [ADP] for shape change and aggregation[1]

Parameter	$[ADP]_{1/2}$ uM	Range of [ADP] Tested uM	IC_{50} nM	$IC_{50}/[ADP]$[3] nM/uM
$V_s(2; 6)$[2]	0.8 ± 0.2	0.7 - 2.5	1.6 - 5.0	2.5 ± 0.4
$PA_3(5; 9)$	1.3 ± 0.2	1.5 - 2.5	1.1 - 3.0	1.0 ± 0.2
$V_a(3; 5)$	2.0 ± 0.1	0.75 - 2.5	0.5 - 1.0	0.5 ± 0.1

[1]Mean ± SD or range of values shown in brackets

[2]Number of donors; with total number of experimental points (n) analyzed indicated

[3]Each IC_{50} determined at a given [ADP] was expressed as nM ZK/uM ADP and averaged for a given donor, with all values pooled for each parameter measured. There was very little intra- and inter-donor variations in these particular experiments

expressed per uM ADP, then their relative values are in the ratio of ~5:2-3:1, as observed in Table 2 for measurements made at one common ADP concentration. It therefore appears that this ratio of ~5:3:1 is observed independently of the [ADP] or analytical procedures used.

Discussion

Previous studies of early ADP-induced platelet recruitment (PA), determined from "singlet" disappearance measured with a haemocytometer (6), and of both shape change (SC) (6,7) and turbidometrically-measured aggregation (TA) (6), have suggested the sequential activation of platelets: unactivated platelets -> SC -> PA -> TA. The rates of SC (V_S) and PA (PA_3) appeared to be similar in magnitude for ADP as activator (7,9), but shape change preceded the onset of PA which showed a lag time of ~1 second (7). A comparison of the ADP sensitivities ($[ADP]_{1/2}$) independently determined for (i) rate of "singlet" recruitment using a haemocytometer (6) and (ii) rate of singlet shape change (20), indicate similar values. However, values of $[ADP]_{1/2}$ determined for the same donors in parallel for rates of PA (PA_3) measured electronically from the decrease in platelet particle number, and of shape change (V_S), show the two processes to have distinct sensitivities (Table 1). We have previously shown that $[ADP]_{1/2}$ determined for PA_3 measured by electronic particle counting is essentially identical to that determined for total particle count using a haemocytometer, but significantly greater than that determined from "singlet" measurements with the haemocytometer (8). Thus, measurements of $[ADP]_{1/2}$ for PA_3 determined from the decrease in platelet particle number, a method generally used in evaluating the dynamics of aggregating colloidal suspensions (22), indicate that for the overall scheme of platelet activation, increasing ADP is

indeed required to drive unactivated platelets -> SC -> PA -> TA. This complements the scheme originally proposed by Holmsen (SC -> TA -> release), with each step requiring increasing stimulus concentrations (10).

Studies of the inhibition of ADP-induced microaggregation (PA_3) were generally made using the electronic particle counter, as was done for determinations of ADP sensitivities. It was shown, using one donor, that very similar IC_{50} values for ZK inhibition of ADP-induced PA_3 were obtained by electronic counting ($[ADP]_{1/2}$ = 1.65 uM) as by haemocytometer counting for "singlet" counts ($[ADP]_{1/2}$ = 1.30 uM): IC_{50} values were respectively 1.10 and 1.25 nM. Our results generally suggest that ZK (Iloprost) differentially inhibits platelet activation, since progressively higher concentrations of ZK were needed to inhibit TA (V_a), PA (PA_3) and SC (V_s), over the range of ADP concentrations tested for activation (Tables 2, 3 and Fig. 4). Though IC_{50} values were generally proportional to the [ADP] tested for any given parameter (Fig. 4 and Table 3), relative IC_{50} values in each of these three parameters values remained on average at about 5:3:1 corresponding to inhibition of shape change (V_s), microaggregation (PA_3) and macroaggregation (V_a). Even when IC_{50} values were compared at the distinct concentrations of ADP driving each activation step at 50% of maximal rate of change (at respective $[ADP]_{1/2}$ values), the differential inhibition persisted (~3:2:1 for V_s:PA_3:V_a). These varied choices of ADP concentrations simulate the variety of conditions reported in the literature in determinations of IC_{50} values for ZK (Iloprost) and other inhibitors (4,11,13,15-18,23, 24), namely the use of high concentrations of activator causing >90% of maximal aggregation response (13,16,23); or the use of varying concentrations chosen to cause comparable changes in different platelet functions being evaluated (11) for the same donor or in a given function for different

donors (15,17,24).

About ten times more PGD_2 is required to inhibit platelet activating factor-induced shape change (SC) than that needed to inhibit turbidometrically measured secondary aggregation and its associated dense granule release (TA-2) (4). This large difference in differential sensitivity is consistent with our observations for ZK as inhibitor where relative IC_{50} values for rates of shape change and macroaggregation (TA) were ~5:1. We would predict even larger differences when comparing the inhibition of shape change to the above TA-2, a more advanced stage in the general activation sequence: SC -> PA -> TA -> TA-2.

Similar studies for IC_{50} values for PGI_2 (Upjohn Co., Kalamazoo, USA) were made for 2 young healthy donors for 2 uM ADP-induced shape change (V_s) and aggregation (V_a) in PRP yielding values of 1.2 $\pm$ 0.3 nM and 2.1 $\pm$ 0.4 nM. Thus, IC_{50} values for PGI_2 or ZK at [ADP] less than that needed to maximally drive TA will have upper values of ~3 nM. It must be noted in this regard that although total platelet cAMP concentration rises by 10-100 times with a log dose-dependence on PGI_2 (25) or ZK (19) concentrations studied with human platelet-rich plasma, there is a threshold requirement for both PGI_2 (19,25,26) and ZK (19) at approximately 3 nM. The range of ZK used in this study was below this proposed threshold, suggesting the absence of any measurable shift in total basal platelet cAMP associated with effective inhibition by ZK or PGI_2 of platelet activation: SC -> PA -> TA. A similar suggestion was previously made in turbidometric studies of the inhibition of SC and TA using a cAMP phosphodiesterase inhibitor (13), and in comparison with the effects of PGE_1 and dibutyryl cyclic AMP (27). However, actual changes observed in cyclic AMP with inhibition may vary with the activator and function examined, as reported for thrombin-induced advanced platelet activation (dense granule release) using PGI_2 and PGE_1 (17),

and with the inhibitor evaluated, as for PGD_2-inhibition of PAF-induced shape change and release (4).

Prostaglandins D_2, E_1, and I_2, as well as dibutyryl cyclic AMP, have all been shown to inhibit calcium mobilization associated with platelet activation (2,3). In particular, PGE_1 which has a platelet receptor common to that of PGI_2 (5) and likely ZK (15-19), can inhibit the rise in platelet intracellular ionized calcium (Ca^{+2}_i) in parallel with inhibition of turbidometrically-measured aggregation (TA) (28). Thus increases in Ca^{+2}_i mediated by ionomycin treatment of platelets had to be about 5- and 20-fold above threshold to cause respectively the appearance of SC (V_s) and of TA (V_a) (30). Such relative increases in Ca^{+2}_i for SC:TA of 1:4 are opposite to that predicted from our IC_{50} values for ZK inhibition of SC and TA. However, the relative importance of Ca^{+2}_i as a second messenger appears to vary according to conditions of activation and inhibition (4,29-32) and according to methods of Ca^{+2}_i detection (31-34). This appears largely associated with the complex interplay between Ca^{+2}_i, other second messengers associated with the phosphoionositide pathway (inositol triphosphate [IP_3] and 1,2-diacylglycerol [DAG]), and cyclic AMP (2,3,31,32). Thus, PGI_2 and cyclic AMP can inhibit Ca^{+2}_i elevation as well as the formation of IP_3 and DAG (2,3,29,31,32) while cAMP can suppress other intracellular components distal to both Ca^{+2}_i and DAG (4,31,32). Therefore, parallel measurements of all of these messengers and associated components such as protein kinases must be made as a function of [Activator] and [Inhibitor] for each step in the activation sequence: shape change -> microaggregation -> reversible macroaggregation -> secondary aggregation and release, to determine their relative and distinct roles in differential platelet activation and regulation.

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Chapter III

Differential Activation and Inhibition of Human Platelets: Direct Comparison of Freshly-Collected Citrated Whole Blood and Platelet-Rich Plasma

Summary

The relative sensitivities of adenosine diphosphate (ADP)-induced activation and of prostaglandin-mediated inhibition were determined for rates of platelet shape change (SC [V_S]) and of early platelet recruitment (PA [PA_3]) for platelets in freshly-collected citrated whole blood. Studies were performed in parallel on the same 3 healthy human donors. The ADP sensitivity, $[ADP]_{1/2}$ ([ADP] giving half maximal rate) was determined for both SC and PA. Distinct ADP sensitivities were obtained from log dose-response studies, with a relative dose dependency for rates of change in the order of $[ADP]_{1/2}$ PA_3 > $[ADP]_{1/2}$ SC by ~2:1. Differential inhibition of SC and PA was evaluated from log-dose response curves for Iloprost (ZK 36 374), a stable carbacyclin analogue of prostacyclin. IC_{50} values corresponding to ZK concentrations causing 50% inhibition of PA [PA_3] and SC [V_S] were found in the relative ratios of 1:~2. In a parallel study citrated whole blood (WB) and autologous platelet-rich-plasma (PRP) were prepared from 10 normal human donors. $[ADP]_{1/2}$ values for PA_3 in WB were shown to be comparable to or, in about half the observations, ~2x greater than that measured for PRP. Independent of an individual donor's sensitivity to ADP, IC_{50} values for PA_3 in WB were similar at all times to values determined in PRP.

Introduction

Platelets display a sequence of responses when adequately stimulated by agonists namely shape change, aggregation, secretion, arachidonic acid liberation and synthesis of prostaglandins and thromboxanes. The extent of these responses will vary with the concentration and potency

of the stimulating agent (1,2). Studies in PRP using ADP as activator have shown the onset of shape change (SC), subsequent early platelet recruitment (PA) and finally macroaggregation (TA) to be distinct processes. This distinction was also observed for inhibition of these processes by a stable prostacyclin analogue, ZK 36374, evaluated in PRP (3,4).

The use of platelet-rich plasma for platelet aggregation studies has at least three disadvantages: 1) the physiological effects of unstable in vivo metabolites may decay over 1-2 hours post bleeding time; 2) because platelets are heterogenous with respect to size, density and metabolic activity (5,6), certain populations of platelets may not be recovered during centrifugation. Indeed, mean % recovery is ~ 67% (7), though the size distribution of residual platelets has not been compared to that of the platelets actually isolated in the PRP; any differential sedimentation of platelets would however favor the loss of larger denser platelets. Therefore, the results of aggregation studies in PRP may not represent platelet function in whole blood; and 3) in PRP, platelet aggregation occurs in the absence of red and white blood cells, thus pre-empting the detection of any possible effects of these cells on platelet function.

Platelet behaviour in whole blood can be very different from that in platelet-rich plasma. Red blood cells in flow or in stirred citrated whole blood are believed to enhance platelet aggregation by releasing 5'-adenosine diphosphate (ADP) (8-11). In fact, the enhancement of such spontaneous, stir-induced platelet aggregation in WB has been proposed as a risk marker for thrombosis (PA at 8 mins of stir) (12). On the other hand white blood cells may inhibit platelet aggregation by releasing prostacyclin (13,14). A significant negative correlation was found between the count of WBC's (polymorphonuclear [PMN] leukocyte) and measured

whole blood platelet aggregation (15). These findings agree with an earlier report by Harrison and colleagues (16), which showed a progressive inhibition of platelet aggregation with the artificial enrichment of PRP with WDC's, using the Born aggregometer.

Studies of platelet aggregation (TA) have been made in fresh citrated whole blood using an electronic whole blood aggregometer (15,17), reported to measure platelet macroaggregation similar to that measured in PRP by turbidometry (15,18). Thus, using one high ADP concentration (20 uM), Abbate et al found much less extensive TA in WB than in PRP (15). A few preliminary studies on selected steps in the activation sequence (e.g. PA or TA, but not both) have been reported for citrated whole blood (18-21). Such PA studies have tended to measure PA at rather long times following addition of activator (1-2 mins) and have not generally been compared with PRP as a function of activator or inhibitor concentrations (19-21). In a direct comparison of PA (slow reaction times) in whole blood versus PRP, the authors claimed that no clear differences could be observed using ADP (1 uM) or epinephrine (1.5 and 0.8 uM); however, no data was provided. The differences seen with collagen were attributed to both chemical and physical contributions of red blood cells, but adhesion was not distinguished from platelet aggregation (21).

Citrated whole blood data for the inhibition of PA at 1-2 mins after 10 uM ADP addition by ZK (19) or by PGI_2 (20,22) yields IC_{50} per uM ADP of 0.05 nM/uM and ~0.3-0.6 nM/uM respectively. This must be contrasted with our result for PRP for ZK of 1.8 $\pm$ 0.1 nM/uM ADP for PA at 10 seconds after 1 uM ADP addition (4). This 30-fold difference for ZK between WB and PRP warrants further investigation. In addition, the stability of prostacyclin (PGI_2) in whole blood and PRP differ: the half-life of PGI_2 in whole blood

is about 50% of that in PRP (~6 vs 11 mins) (23). We therefore chose to use a more stable PGI_2 analogue, ZK36374, for a comparison of platelet behaviour in WB and PRP (4).

Recently we reported on the dynamics and sensitivity for activation and inhibition of the overall sequence of platelet functional events determined in PRP (SC -> PA -> TA) (4; reported in Chapter 2). Using electronic particle counting in parallel with optical measurements we now report the differential ADP sensitivity ($[ADP]_{1/2}$) determined in citrated whole blood for the sequence of activation steps: unactivated platelets -> SC -> PA. We then evaluate the relative amounts of ZK needed to inhibit this sequence (IC_{50} values) using an [ADP] $\geq$ the respective $[ADP]_{1/2}$. Finally, we compare the pharmacological sensitivities to activator and inhibitor for SC and PA determined in parallel for citrated whole blood and autologous PRP.

Materials and Methods

Preparation of Citrated Whole Blood (WB) and Platelet-Rich Plasma (PRP)

Normal donors were chosen from healthy males and females between the ages of 20 and 50 years. Ingestion of aspirin or any other non-steroidal anti-inflammatory drugs was prohibited at least 2 weeks prior to donation. Whole blood was drawn by venipuncture into 3.8% sodium citrate (one volume to 9 volumes blood), after discarding an initial collection of 3 ml, and kept in polypropylene tubes in 50 ml aliquots at 37°C (herein referred to as WB); PRP was prepared at 37°C and pH = 7.4 $\pm$ 0.1 as previously described (24). The hematocrit was determined by centrifuging the freshly drawn whole blood in a heparinized microcapillary tube (75 by 1.1 to 1.2 mm inner diameter; Fisher Scientific

Co., Pittsburgh, Pa.) in a micro-centrifuge (Autocrit; Clay-Adams, Inc., Parsippany, N.J.) for 5 minutes; duplicates varied by < 3% from mean hematocrit values (3).

Aggregation from Platelet Counting

Platelet microaggregation (PA) was estimated from the percent decrease in the particle count determined with an electronic (resistive) particle counter as previously described (18). A 0.1 ml volume of PRP was placed in a glass cuvette (6.9 mm x 45 mm) and stirred in an aggregometer at 1000 rpm (24). For whole-blood studies, to minimize any spontaneous platelet aggregation, a 0.1 ml suspension was stirred for < 5 seconds prior to the addition of ADP. Aliquots were transferred before each measurement (6 samples at a time) with a polypropylene-tipped volumetric syringe into glass cuvettes. It was not found advantageous to use siliconized cuvettes in any of the studies herein presented. At selected times following the addition of activator to PRP or WB, 0.5 ml of 0.8% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) was added directly to the cuvette, and counted as previously described (18). The fixed WB samples were spun in a desk-top centrifuge at 150 x g for 2 minutes to yield fixed PRP used to count platelets. Twenty microliters of this supernatant was immediately dispensed into 10 ml of Hematall isotonic diluent, and the platelet particle count was determined by an electronic particle counter (18). The percent platelet aggregation, PA_t, was calculated from the following relation:

$$\%PA_t = [1-N_t/N_o] \times 100\%$$

where N_t, N_o are respectively, the platelet particle (electronic) counts at times t and zero. PA was measured at

3 seconds (PA_3) corresponding to the initial linear rate for early platelet recruitment; time courses were obtained for 2 donors for both WB and PRP and found to be indistinguishable from each other and as previously published for PRP (3,18). The [ADP] required to induce one-half maximal rate of PA_3, $[ADP]_{1/2}$, was readily determined from a log dose-response plot of $PA_3/(PA_3)max$ versus [ADP] (Fig. 2). $[ADP]_{1/2}$ values were routinely obtained from PA at 3 seconds in PRP and WB, measured for [ADP] from 0.2 to 10 uM. These concentrations in whole blood represent actual plasma concentrations, corrected for donors hematocrit. Maximal values of PA_3 were obtained for an absolute plasma [ADP] = 10 uM.

Shape Change Measured Turbidometrically in PRP

ADP (1-10 ul) was quickly injected using a Hamilton syringe (Hamilton Co., Reno, Nev.) into 0.4 ml of PRP in a cuvette (6.9 mm x 45 mm) with stir bars (6mm x 1 mm) spun at 1000 rpm, 37°C. Changes in light transmission were monitored using an aggregometer (Payton dual channel aggregation module), with a rapid chart speed as previously described (4,24). The slope of the initial decrease in light transmission, V_s, reflects the rate of platelet shape change in the presence or absence of inhibitor, as previously described (4,25). Maximal values of V_s were obtained for [ADP] = 10 uM. The [ADP] required to induce one-half maximal rate of SC (V_s), [ADP]1/2, was determined from log dose-response plot of $V_s/(V_s)max$ versus [ADP] in the range of 0.1-10.0 uM.

Inhibitor Studies with Iloprost (ZK 36 374)

1. Morphometric Shape Change and Early Platelet Recruitment

ZK (Iloprost, ZK 36 374) was added to PRP or WB (< 5% v/v PRP or WB); at a final absolute concentration ranging from 0.5 to 10 nM). It was added at 37°C, with < 1 sec stir, followed by a one minute incubation (no stir) prior to the addition of ADP. ZK, with one minute incubation in PRP or in washed platelets, has been reported to be 2.5 to 10 times more potent than prostacyclin (PGI_2) in elevating basal cAMP levels in human platelets (26,27). In whole blood, stirring was kept to < 5 seconds before the addition of inhibitor to minimize any stir-induced platelet activation. With such minimal stirring, the % Discocytes (D) in WB was found to be constant over the 15-20 minutes post-bleeding required to complete the studies (Mean: 60 ± 10% for 4 different donors). ZK over a 0.5-10.0 nM range did not cause any significant direct shape change when incubated with WB and treated with Tyrodes exactly as described for evaluations of IC_{50} for ADP.

ZK concentrations causing 50% inhibition of PA_3 and V_a (IC_{50} values), were derived from a plot of % inhibition calculated from $PA_3/(PA_3)max$ or $V_s/(V_s)max$ versus [ZK 36 374] determined for a range of ADP concentrations.

2. Bioassay for effective plasma concentration of ZK in whole blood

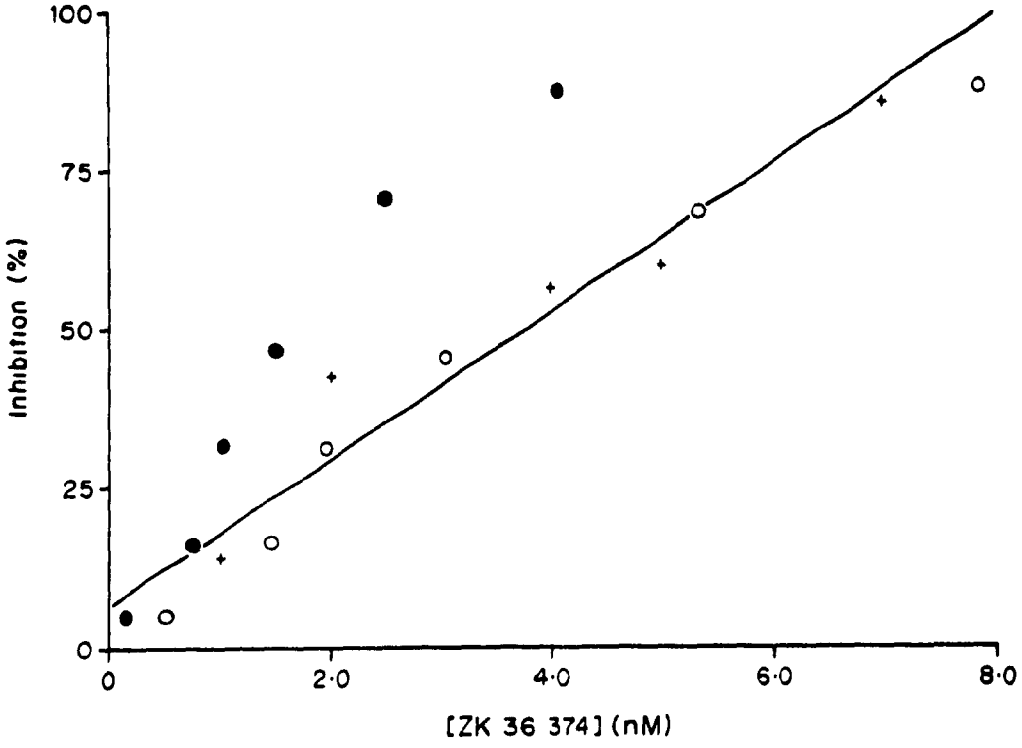
Citrated whole blood (WB) and PRP were prepared as above. Platelet-poor plasma (PPP) was prepared by centrifuging PRP for 10 minutes at 1450 x g or for 1-2 mins at 10,000 x g on an Eppendorf Microfuge (Beckman Instruments, Inc. Palo Alto, Ca.) and allowing it to

incubate at 37°C for an additional 30 minutes before use to remove residual ADP (24). ZK was incubated in a 0.1 ml suspension of PRP and PPP (1:1) at 37°C in an aggregometry cuvette with stirring at 1000 rpm for one minute prior to the addition of ADP (4). The final concentration of ZK ranged from 0.5 to 10 nM. A standard curve of % inhibition versus [ZK] was obtained for PA_3 determined at 1.5 uM ADP (Fig. 1). To determine the plasma concentration of ZK added to WB, ZK was added to a separate tube of 10 mls of citrated WB (10 nM) and incubated for 10-15 minutes. PPP_{ZK} was prepared by centrifuging the WB for 10 minutes at 1450 x g (incubation followed for 30 minutes). Varying volumes of PPP and PPP_{ZK} (totalling 1.0 ml) were added to a 1.0 ml sample of PRP. The [ZK] in plasma was thus diluted giving a final concentration range of 0.25-8.0 nM (based on the WB volume). The [ZK] in PPP_{ZK} so isolated was measured with the above assay (Fig. 1) and shown to correspond to no uptake into red/white blood cells (repeated for 2 donors). Thus, the effective [ZK] in whole blood is expressed as the concentration in plasma corrected for Hematocrit, as done for non-permeating [ADP]; uncorrected data is shown only in Fig. 3.

Dose-Response Studies of Shape Change Measured Microscopically in Whole Blood

The rate of platelet shape change was estimated as previously described (4); ADP was added to WB stirred at 1000 rpm, 37°C, as for aggregation studies above. The suspension was mixed for < 1 second to ensure complete mixing and minimal aggregation. Stirring prior to the addition of ADP was < 5 seconds to ensure minimal spontaneous platelet activation and aggregate formation. At different times following ADP addition (2-60 seconds), all morphological changes were arrested with 0.8% glutaraldehyde

Fig. 1 A standard curve of % inhibition versus ZK added to PRP for PA_3 at 1.5 uM ADP, for a single donor, is used to determine the plasma concentration of ZK in WB. Crosses (+) represent data for the standard curve determined in PRP. Closed circles (●) depict the observed % inhibition for ZK added to WB when expressed per unit volume of WB (see Methods), and by open circles (O) when expressed per unit volume of plasma when corrected for the donor's hematocrit (=52%).



(0.5 ml/0.1 ml WB) (28). The choice of 5 seconds was confirmed as appropriate for measuring the initial linear rate of platelet shape change as previously reported for PRP (28). To look at platelet shape change in whole blood, "PRP" was isolated by spinning the fixed WB samples on a desk-top at 150 x g for 2 minutes (International Equipment Co., Needham Hts, Mass). Slides were prepared as previously described (29). For inhibitor studies, WB was pre-incubated with ZK (< 1 sec. stir) for one minute as above.

Platelet Morphology

Platelet morphology in whole blood was determined directly by phase contrast microscopy (400x) as previously described (28). The percent of echinocytes (shape-changed platelets) distinct from unactivated smooth-surfaced discocytes, was determined by examining 200 platelets. Discocytes "D" are unactivated discs as previously described, including those discocytes containing only 1 pseudopod (~ 30 $\pm$ 10% of all D's so counted), since such D's still have a mean axial ratio (r_p) close to that of smooth-surface D's ($\leq$ 0.35) (30). The reproducibility of platelet morphology determined by the above criterion was to within 3% when measured twice by the same investigator and < 15% between two investigators in the same laboratory counting the same sample.

Materials

Trisodium citrate (J.T. Baker Chemical Co., Phillipsburg, N.J.), and ADP (Sigma Chemical Co., St. Louis, Mo.) were prepared in Ca^{2+}/Mg^{2+} free Tyrodes buffer as previously described (31) and kept frozen (-20°C) as a stock solution. ZK (Iloprost; ZK 36 374), was a gift from Dr. Th., Krais, Schering AG, Berlin, West

Germany. It was prepared as a frozen (-20°C) 10 uM stock solution in saline (0.9% NaCl and 0.125% sodium bicarbonate, pH = 8.2). Such ZK solutions were used within 6 months; solutions older than 1 year were found to cause direct activation (SC) of platelets in control studies. Glutaraldehyde E.M. Grade (8% stock in sealed ampoules (Polysciences Inc., Warrington, Pa.)) was prepared in modified Tyrodes (Ca^{2+}-Mg^{2+} free) buffer. Final vehicule concentrations for both ADP and ZK were < 5% v/v in PRP and WB.

Results

ADP Sensitivities for PA_3 in WB versus PRP

Stir-induced spontaneous platelet aggregation (SIPA) in WB was seen on average to be as high as 19% by 30 secs of stir, 31% by 60 secs and 52% by 15 minutes. These values were highly variable between 30 secs to 15 mins, being < 5% in 30-50% of observations, including the same donor studied on different occasions. No significant differences in SIPA over 30 secs to 15 mins were found when comparing normal versus siliconized glass cuvettes (31). However, no significant PA (< 5%) was observed in all control studies where WB was stirred in total for < 5 seconds over the < 20 minute period used in evaluating platelet function.

A typical log dose-response curve for the rate of ADP-induced aggregation measured by electronic particle counting (PA_3), is shown for a single donor in Fig. 2 for WB. Table 1 summarizes the ADP sensitivities for PA_3 determined from such curves for 10 donors. The ADP sensitivities for PA_3 were observed to fall into two categories: Type 1, where sensitivity for PA_3 in WB was no different from that seen in

Table 1 Comparison of Sensitivities for Rates of Aggregation (PA_3) in Citrated WB and PRP

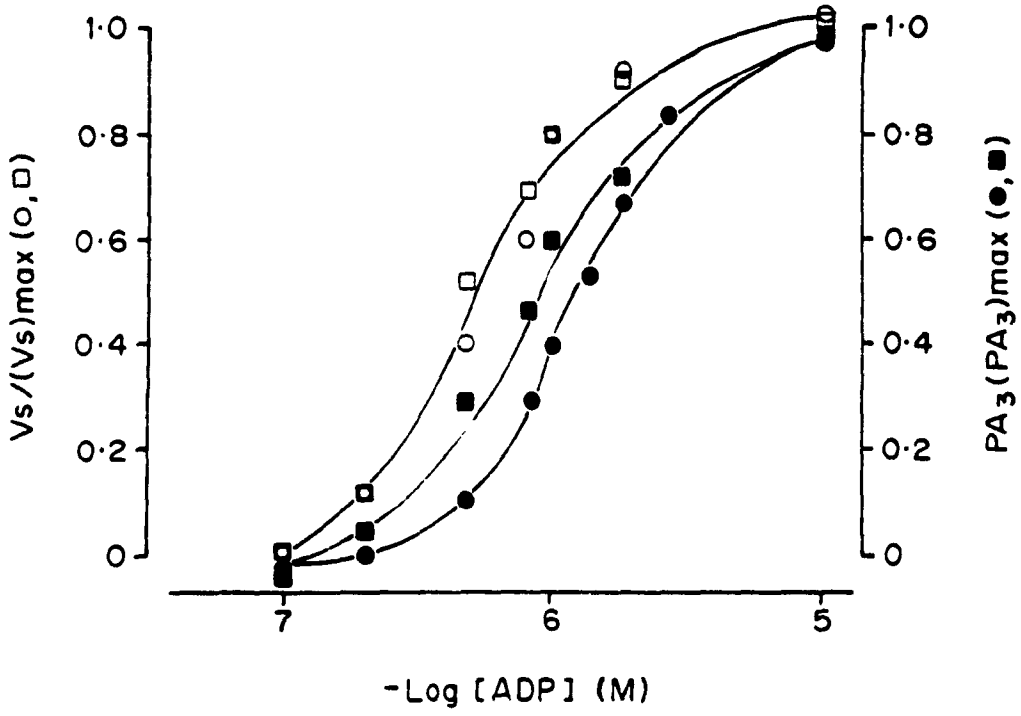
Donors	$[ADP]_{1/2}$ Values for PA_3 for WB	PRP
	uM	
Type 1 (8)[a]	1.2 ± 0.3[b] (0.9-1.6)[c]	1.1 ± 0.2 (0.8-1.4)
Type 2 (4)	2.6 ± 0.1 (2.5-2.7)	1.1 ± 0.4 (0.6-1.6)

[a]# of different donors shown in brackets. Three donors were common to both types of observations.

[b]Mean ± S.D for all donors. Data in WB has been corrected for Hematocrit.

[c]Range of values shown in brackets.

Fig 2. Log dose-response curves for ADP-induced rates of shape change (V_S), and early platelet recruitment (PA_3) in WB for 2 donors. Typical curves are shown for relative changes in PA_3 with one donor displaying type 1 behaviour (■) and the other type 2 (●) (see Table 1). Shape change (V_S) is represented by open symbols (○, □). Results are shown uncorrected for the donor's hematocrit, both = 43%.



PRP obtained from the same donor; and Type 2, where the platelets appeared to be up to 4x less sensitive to ADP in WB than when analyzed in the same donor's PRP. In fact, a ratio of $[ADP]_{1/2}$ values for WB to PRP determined for each donor and then pooled gave WB:PRP values of 1.2 $\pm$ 0.3 ($p<0.005$) and 2.6 $\pm$ 0.9 ($p<0.01$) respectively for types 1 and 2.

The ADP sensitivities obtained in WB for three individual donors examined on two separate occasions were found to fall within both types 1 and 2 on these different occasions. Variations in PA_3 values between two different fixed samples from the same platelet suspension were typically <7% from the mean, whether determined for PRP or WB. This was found to translate into variations for $[ADP]_{1/2}$ for PA_3 typically <11% from the reported mean values.

ADP Sensitivities for SC versus PA_3 in WB

The relative ADP dose dependency for platelet activation in WB is ~2:1 for PA versus SC (Table 3 and Fig. 2). It appears that about two times more ADP is required to drive early platelet recruitment into microaggregates than needed for shape change as previously reported for PRP (4).

ZK Sensitivities for Inhibition of PA_3 in WB versus PRP

Similar ZK sensitivities (IC_{50}'s) were seen on average, for inhibition of PA_3 in PRP or WB, irrespective of differences seen in ADP sensitivity for types 1 & 2 (Table 2). Thus, type 1 & 2 donors were on average indistinguishable with respect to IC_{50} (Table 2). As the [ADP] used for any given experiment was not constant, we had to consider its effect on IC_{50} values which were previously shown in similar studies with PRP to vary linearly with the

[ADP] used for any given parameter, particularly PA_3 (Fig. 4 in Chapter 2). We therefore calculated the IC_{50} values for PA_3 shown in Table 2 for each donor, expressed as nM ZK per uM ADP used, and then pooled this data. We still found IC_{50} values for types 1 and 2 donors to be identical, respectively 0.9 $\pm$ 0.5 and 0.9 $\pm$ 0.4 nM ZK per uM ADP. Slightly higher values of 1.2 $\pm$ 0.3 nM ZK per uM ADP were obtained for PRP (n = 9 donors). When we compared these normalized ZK values for WB versus autologous PRP (for the same donor), we again found no difference in the ratio for type 1 (n = 5) and type 2 (n = 3) donors, respectively 0.7 $\pm$ 0.2 and 0.7 $\pm$ 0.5.

Inhibition of PA_3 versus SC in WB

Log dose-response curves were obtained for ZK-mediated inhibition of V_S and PA_3 conducted in parallel at a common ADP concentration sufficient to drive these uninhibited processes by > 50% of their maximal rates. Microscopically-measured rates of shape change (V_S) and aggregation (PA_3) in WB were increasingly inhibited with a progressive increase in ZK (Iloprost) added for 1 minute preceeding ADP addition (stir time < 1 sec for ADP and ZK). A typical set of such curves is shown in Fig. 2 for 1 donor; ZK concentrations causing 50% inhibition (IC_{50}) of V_S and PA_3 are derived from such curves. Results for such studies show relative IC_{50} values for V_S and PA_3 to be in the order of 2:1 (Table 3). This suggests that ~ 2 times more inhibitor is required to comparably inhibit V_S than PA_3.

Activation and Inhibition of SC in WB versus PRP

Finally activation of platelet shape change (SC) by ADP, and its inhibition by ZK were each directly compared in

Table 2 ZK Sensitivities (IC50's) for Inhibition of Rates of Platelet Aggregation (PA_3) Measured in Citrated PRP and WB, at an ADP concentration > [ADP]1/2

Platelet Suspension	Donors	$[ADP]_{1/2}$ uM	[ADP] used uM	ZK-IC_{50} nM
PRP	One type (9:18)[a]	1.2 ± 0.3 (0.6-1.6)	1.7 ± 0.5[b] (1.0-2.5)	2.0 ± 0.7 (0.7-3.4)
WB	Type 1 (9;12)	1.2 ± 0.3 (0.6-1.6)	2.9 ± 1.2 (1.1-5.3)	2.6 ± 1.4 (1.1-5.3)
WB	Type 2 (10;11)	2.5 ± 0.3 (2.0-3.2)	2.9 ± 0.7 (1.9-4.4)	2.6 ± 1.2 (1.0-4.8)

[a]Data for n donors; on x occasions (n;x).

[b]Mean ± S.D, and range of values shown in brackets. Data in whole blood was adjusted according to donor's Hct.

Table 3 Relative ADP ([ADP]1/2) and ZK ([IC]50) sensitivities for Rates of Platelet Shape Change and Aggregation in Citrated Whole Blood[a]

	[ADP]1/2	Relative [ADP]1/2[b]	[ADP] used[c]	$[IC]_{50}$	Relative $[IC]_{50}$[d]
	uM		uM	nM	
Shape Change					
Vs	0.8 ± 0.3 (0.4-1.1)	1.0	1.1 ± 0.1 (1.0-1.2)	3.5 ± 0.4 (3.0-3.8)	1.8 ± 0.1 (1.7-1.9)
Aggregation					
PA3	1.5 ± 0.8 (0.7-2.4)	1.9 ± 0.1 (1.8-2.1)	1.8 ± 0.7 (1.4-2.6)	2.0 ± 0.3 (1.7-2.3)	1.0

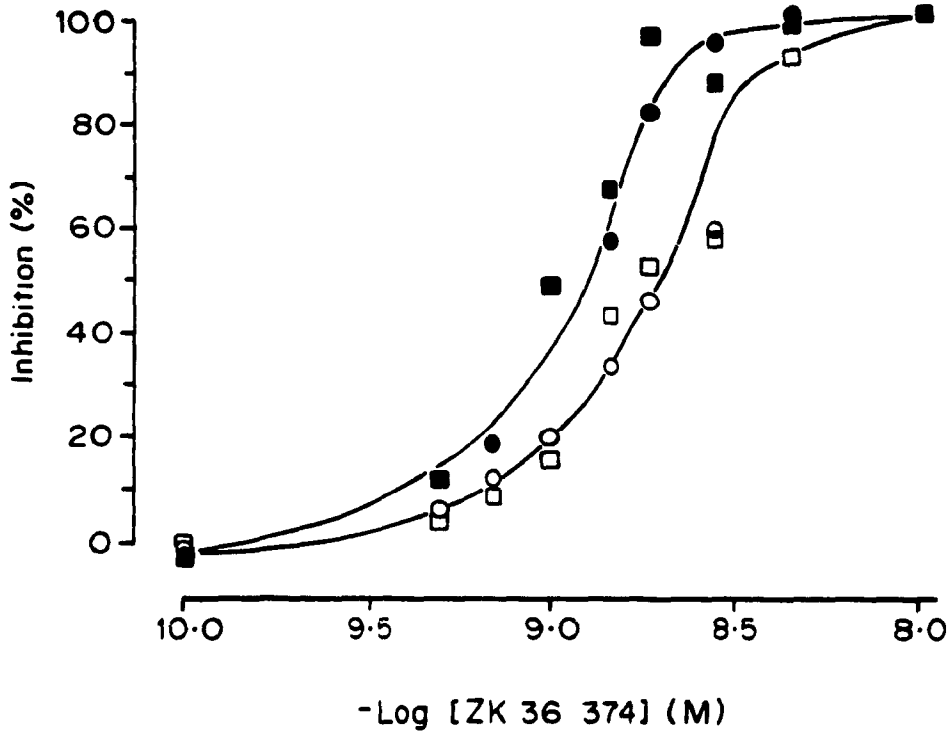
[a]Data for 3 donors (2 male, 1 female) expressed as Mean ± S.D, with range of values shown in brackets. All donors were type 2, except for 1 male. All data has been corrected for hct.

[b]The [ADP]1/2 values for the two parameters were compared relative to Vs normalized to 1.0 for each donor, and then averaged for all the donors. Significance of differences (paired t-test) for each parameter pair was $p < 0.005$.

[c][ADP] used for PA3 was approximately one-half to two-times the [ADP]1/2, while for Vs the ADP used on occasion was twice the [ADP]1/2.

[d]The IC50 values for the two parameters were compared relative to PA3 normalized to 1.0 for each donor, and then averaged for all the donors. Significance of differences for each parameter pair was $p < 0.005$.

Fig 3. Log dose-response curves for ZK-mediated inhibition of ADP-induced rates of microaggregation (PA_3) (●, ■) and shape change (V_S) (○, □) are shown for the same 2 donors as seen in Fig 2. The concentrations of ADP used were 0.7-0.8 uM, except that 1.5 uM ADP was used for PA_3 for donor (■).



parallel for WB and autologous PRP samples. Mean $[ADP]_{1/2}$ for shape change (V_s) so measured were comparable for WB and PRP, as were $[ZK]_{50}$ values (Table 4).

Discussion

It has been suggested that ADP-induced platelet macroaggregation in whole blood requires larger amounts of ADP than in PRP (15), ascribed to the presence of white and red blood cells whose plasma membranes are rich in ADP'ase and 5-nucleotidase activity (32,33). However, others believe that red blood cells enhance platelet aggregation by releasing ADP (8-11) while white blood cells reduce platelet aggregation by generating PGI_2 (13,14). In view of the therapeutic potential of prostaglandins like PGI_2 and ZK 36374 in the control of thrombo-embolic disorders (1,2,4,14,19,20,25-27), it is important to determine any differences in their behaviour in whole blood versus platelet-rich plasma. We have therefore conducted direct parallel evaluations of platelet activation and inhibition in WB versus PRP, and have compared the differential sensitivities for SC and PA_3 in WB, as previously reported for PRP (4).

We found that early platelet activation measured from the rate of shape change, and its inhibition by ZK, were no different when compared for citrated whole blood or PRP (Table 4). Inhibition of SC requires > 2x the concentration of inhibitor needed to inhibit early microaggregation (PA_3; Table 3), and likely 3-5x more than needed to inhibit macroaggregation as reported from studies in PRP (4). Therefore, the presence of small amounts of "natural" inhibitors in fresh WB, even at levels capable of significantly inhibiting aggregation (PA or macroaggregation), would not be expected to affect $[ADP]_{1/2}$ values for SC in WB or PRP.

Table 4 A comparison of ADP and ZK Sensitivities for Rates of Shape Change (V_s) Measured in WB versus autologous PRP.

Donor	Sensitivities for Shape Change (V_s)			
	$[ADP]_{1/2}$ for		ZK-IC_{50}[a] for	
	WB	PRP	WB	PRP
	uM		nM	
1	1.1	0.7	3.8	2.3
2	0.5	0.6	3.0	2.7
3	0.8	0.5	3.6	3.3
$\bar{X}\pm$SD	0.8 ± 0.3	0.6 ± 0.1	3.5 ± 0.4	2.8 ± 0.5
Range	(0.5-1.1)	(0.5-0.7)	(3.0-3.8)	(2.3-3.3)

[a]IC_{50} values for Shape Change (Vs) were tested at [ADP] = 1.1 uM and 2.0 uM in WB and PRP respectively. All values in whole blood represent concentrations corrected for hematocrit.

Sensitivity differences for rates of ADP-induced platelet recruitment (PA_3) were observed for different donors on different occasions in WB (types 1 & 2) but not in autologous PRP. Thus, in ~50% of observations in WB, ~2x more ADP was required to yield similar rates of PA (type 2) as observed in autologous PRP or in type 1 occasions in WB (see Table 2). This suggests that platelets may be more refractory in freshly-collected whole blood (less sensitive to ADP) for some donors on any particular occasion (type 2) when compared to PRP isolated in a slower step from the WB. This apparent hyposensitivity in fresh WB (within 30 minutes of blood collection) may arise from the effects of PGI_2 and/or other natural inhibitors such as EDRF or nitric oxide (34) present at more elevated concentrations in WB from donors showing type 2 behaviour. It appears that such 'inhibitors' and their effects on platelets would decay sufficiently rapidly that their effect is no longer measurable in PRP prepared from WB exhibiting this type 2 behaviour.

It is unlikely that the natural inhibitor in type 2 WB is primarily PGI_2 as no significant difference was found in IC_{50} for ZK between types 1 & 2 observations (Table 2). It would have to be another inhibitor which does not affect the $[ADP]_{1/2}$ measured for PA_3 in PRP (i.e. decays sufficiently rapidly), and which acts independently of inhibitory pathways used by PGI_2, believed to share the same receptor as ZK (26,27).

In freshly citrated whole blood, ~2x more ADP was needed to cause 50% of the maximal rate for PA_3 than that needed to drive SC; this is comparable to reports for similar studies in PRP (4). Experiments were done for two type 1 donors and one type 2 donor. In contrast, ~2x more ZK was needed to inhibit SC than PA_3. Previous studies in PRP show that the relative sensitivities using ADP for SC and PA_3 are also in the relative ratios of ~2:1 (4).

It has been reported that PGI_2 and PGE_1, respectively at 5-24 nM and 85-338 nM, can cause direct and major increases in the % of human platelets containing pseudopods within 10 secs of addition of these agents to human PRP, with immediate decreases in %T associated with shape change seen in the aggregometer (35). The use of glycine buffer at pH = 10.5 at 4% by volume addition by Zilla et al (35) is worrisome, with a very significant slow and time-dependent increase in %T seen for addition of the glycine buffer alone. This is suggestive of abnormal platelet 'swelling' or a change in the membrane refractive index (31). Indeed, these authors suggest that PGI_2 or PGE_1 may induce partial release from the alpha-granules and cause partial SC. These results are in contrast to our control studies with the stable PGI_2 analogue, ZK, where we found no evidence for any direct shape change activation for a 0.5-10 nM range of concentrations, whether evaluated for PRP or WB.

Effective exclusion of ZK from red blood cells was determined from bioassay studies (Fig. 1 and Methods). Thus, ZK primarily remains in the plasma compartment, associated for example with albumin, rather than being taken up primarily by the red blood cells. The exclusion from platelets also shows that less than ~5% of externally-added ZK appears to be taken up by receptors on the platelet surface; this would suffice for activity as suggested by observations of ADP acting on platelets (28). Thus the ZK, likely acting at a platelet surface PGI_2 receptor (26,27), need not enter platelets nor any other blood cells. As the inhibitory effects of ZK in whole blood were evaluated at a high concentration (10nM in WB), it seems unlikely that any uptake of ZK by red blood cells would be counterbalanced by production by certain white blood cells (13,14). The observed compartmentalization of ZK in plasma, similarly expected for PGI_2 with nearly identical structure to ZK (36), ensures that it remains optimally available to

platelet surfaces in the presence of > 100x the cell volume occupied by the red blood cells present in blood.

Our results cannot exclude some involvement of ADP released by the red blood cell or prostacyclin from the white blood cell (8-12). However, we do show that the sensitivities for activator (ADP) and inhibitor (ZK) in PRP and WB are essentially the same in measurements of rates of shape change. Results for measurements of early, rapid rates of platelet recruitment into microaggregates (PA_3) show some variability for activation in WB, but none in PRP, and none for inhibition in WB vs PRP. Possible differences in WB versus PRP for measurements of macroaggregation (15) remain to be determined.

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CHAPTER IV
JOINT SUMMARIES AND CONCLUSIONS

In PRP, using electronic particle counting [1], in parallel with turbidometric measurements [2], we studied the differential ADP sensitivity ($[ADP]_{1/2}$) for the sequence of unactivated platelets -> SC -> PA - > TA. We then evaluated the relative amounts of ZK needed to inhibit this sequence using a common [ADP] for all three steps in the activation sequence or a [ADP] corresponding to respective $[ADP]_{1/2}$ values determined for each activation step.

In WB, the relative sensitivities of ADP-induced activation ($[ADP]_{1/2}$) and of Iloprost (ZK)-mediated inhibition ($ZK\text{-}IC_{50}$) were determined for rates of platelet shape change (SC [V_s]) and of early platelet recruitment for platelets in freshly collected citrated whole blood. These measurements were also made in parallel for autologous preparations of PRP in order to determine whether plasma or cellular components present in freshly collected WB (but not present in PRP), alter platelet function. The experimental results are contained in Chapters II and III.

Chapter II

1. The relative dose dependency for platelet activation by adenosine diphosphate (ADP) is in the order of TA > PA > SC; approximately in the ratio of 1:3:4. It appears that progressively larger concentrations of ADP are required to drive platelet shape change, early recruitment, and build-up of larger aggregates.

2. Log dose-response curves were obtained for ZK-mediated inhibition of V_s, PA_3 and V_a conducted in parallel at a common ADP concentration sufficient to drive all these uninhibited processes by $\geq$50% of their maximal rates. Results for such studies obtained at a common [ADP] show

relative IC_{50} values for V_s, PA_3 and V_a to be ~5:~2:1. This suggests that ~5 and ~2 times more inhibitor is required to comparably inhibit V_s and PA_3 than V_a respectively, when measured at one common ADP concentration (~1.5 uM).

3. IC_{50} values for ZK were also determined at the $[ADP]_{1/2}$ value for each parameter. We again observed a differential sensitivity to ZK as inhibitor, with ~3 and ~2 times more ZK needed to inhibit rates of shape change (V_s) and microaggregation (PA_3) than that required to inhibit macroaggregation (V_a).

4. Turbidometrically measured rates of platelet shape change (V_s) as a function of ZK-inhibition do not have artifacts associated with platelet volume or refractive index changes [3]. Log dose-response curves for ZK-inhibition of V_s induced by 1.5 uM ADP by direct microscopy were essentially identical to those obtained by turbidometry.

5. A linear correlation for rates of shape change (V_s), microaggregation (PA_3) and macroaggregation (V_a), with relative slopes of 5.1:2.7:1 was observed between IC_{50} values and the particular [ADP] tested. It therefore appears that the ratio of ~5:3:1 is observed independently of the [ADP] or analytical procedures used.

Chapter III

1. Stir-induced spontaneous platelet aggregation (SIPA) in whole blood was seen on average to be as high as 19% by 30 secs of stir, 31% by 60 secs and 52% by 15 minutes. When stirring time was controlled (<5 secs), no significant PA

was observed over the < 20 minute period used to evaluate platelet function.

2. The ADP sensitivities for microaggregation (PA_3) in WB were observed to fall into two categories: Type 1, where sensitivity for PA_3 in WB was no different from that seen in PRP obtained from the same donor; and Type 2, where the platelets appeared to be up to 4x less sensitive to ADP in WB when analyzed in the same donor's PRP.

3. The ADP sensitivities for PA_3 in WB for individual donors on different occasions were found to fall within both categories.

4. The relative ADP dose dependency for rates of platelet shape change (V_s) and microaggregation (PA_3) is ~2:1. It appears that about two times more ADP is required to drive early aggregate formation than needed for shape change.

5. Similar IC_{50}'s were seen for PRP vs WB, irrespective of differences in ADP sensitivity for types 1 & 2 donors.

6. The relative ZK dose dependency for the inhibition of rates of platelet shape change (V_s) and early platelet recruitment (PA_3) was shown to be in the order of 2:1. This suggests that ~2x more inhibitor is required to comparably inhibit V_s than PA_3.

Conclusions

This thesis verified original ideas concerning platelet functional events. It expands on Holmsen's classic scheme for platelet activation (4) based on studies focused on turbidometrically-measured shape change (SC), primary aggregation (TA-1), secondary aggregation (TA-2) and associated release reaction, with each step requiring increasing stimulus concentrations (4): namely, SC -> PA -> TA-1 -> release and TA-2.

It further dealt with the dynamics and sensitivities for inhibition of this overall sequence of activation events.

Differences in ADP sensitivities ($[ADP]_{1/2}$) in whole blood versus platelet-rich plasma were seen 50% of the time, for measurements of early platelet recruitment (PA_3). No differences in WB vs PRP were seen for $[ADP]_{1/2}$ and $[ZK]_{50}$ values for platelet shape change (SC). The differences in the ADP sensitivities for PA_3 seen in WB vs PRP, may be attributable to the presence of inhibitors such as nitric oxide present in freshly collected whole blood. This remains to be further investigated.

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