MECHANISMS FOR PLATELET HYPERACTIVITY AND ABNORMAL CALCIUM HOMEOSTASIS IN DIABETES MELLITUS

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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SUMMARY

Platelet hyperactivity has been suggested to contribute to the pathogenesis of diabetic vascular complications (macro and microangiopathy) which are the leading cause of morbidity and mortality in diabetic patients. However the mechanisms underlying platelet hyperactivity remain unclear. The overall objective of this thesis is to determine the mechanisms responsible for platelet hyperactivity in diabetes mellitus.

Cytosolic Ca^{2+} is a crucial second messenger in platelet activation, including shape change, adhesion and aggregation. The *first part* of the study was designed to investigate whether platelet Ca^{2+} homeostasis is deranged in diabetes and thus may be responsible for platelet hyperactivity. Platelet cytosolic free Ca^{2+} concentration and aggregation were simultaneously measured in a Jasco fluorimeter. Platelets were obtained from a group of metabolically uncontrolled diabetic patients and from a group of normal healthy subjects. HbA_{1c} level was used as an index of chronic glycemic control. Both the basal cytosolic Ca^{2+} concentration and agonist (thrombin and collagen)-stimulated $[Ca^{2+}]_i$ responses were enhanced in platelets from diabetic patients. The contents of Ca^{2+} in the platelet intracellular store (DTS) and the ability to release Ca^{2+} from DTS after thrombin stimulation did not differ in these 2 groups of subjects. The sequestration or extrusion processes of cytosolic Ca^{2+} were impaired in platelets from diabetics.

The purpose of *the second part* of the study was to determine the possible mechanisms for abnormal platelet calcium homeostasis in diabetes, mainly focusing on the plasma membrane Na^+-Ca^{2+} exchanger. In the first series of experiments, the existence and physiological role of the Na^+-Ca^{2+} exchanger in platelets from normal subjects was investigated. The specificity of the Na^+-Ca^{2+} exchange blocker CBDMB (an amiloride analogue) was tested in intact platelets and was found to inhibit the Na^+-Ca^{2+} exchanger have no effect on the Na^+-H^+ exchanger at μM concentrations. Three different approaches were used to study the Na^+-Ca^{2+} exchanger:(1). reduction in the transmembrane Na^+ gradient; (2). use of CBDMB and (3) XIP, a structurally different inhibitor of the Na^+-Ca^{2+} exchanger. Similar results were obtained with both inhibitors. In platelets from normal subjects, the Na^+-Ca^{2+} exchanger works in the forward mode, mediating Ca^{2+} efflux in the resting state and after thrombin stimulation, however following collagen stimulation the Na^+-Ca^{2+} exchanger works in the reverse mode.

In the second series of experiments, the sensitivity to CBDMB was utilized to study the direction and activity of the Na^+-Ca^{2+} exchanger in intact platelets and comparisons were made between platelets from normals and diabetics. In normal subjects, CBDMB increased resting $[Ca^{2+}]_i$ suggesting that the Na^+-Ca^{2+} exchanger works in the forward mode (calcium efflux) whereas in diabetes, CBDMB had no effect on resting $[Ca^{2+}]_i$ indicating that the activity of the forward mode is blunted. After thrombin activation, CBDMB increased the second phase of the thrombin-induced $[Ca^{2+}]_i$ response in normals,suggesting that the Na^+-Ca^{2+} exchanger works in the forward mode mediating calcium efflux. In platelets from diabetics, CBDMB decreased the thrombin-induced

$[Ca^{2+}]_i$ response indicating that the Na^+-Ca^{2+} exchanger works in the reverse mode mediating calcium influx. In platelets from normal subjects, CBDMB decreased the collagen-stimulated $[Ca^{2+}]_i$ response suggesting that the Na^+-Ca^{2+} exchanger works in the reverse mode. In diabetics the decrease in $[Ca^{2+}]_i$ following CBDMB in collagen-activated platelets was enhanced indicating that the activity of the exchanger in the reverse mode was increased. These results indicate that in diabetes the direction and activity of the Na^+-Ca^{2+} exchanger are altered and may be one of the mechanisms for the increased platelet cytosolic calcium and hyperactivity seen in diabetes.

The *third part* of the study evaluated the direct effect of hyperglycemia on platelets in vitro. We found that high glucose (45 mM) had no acute effect on thrombin and collagen-induced increases in $[Ca^{2+}]_i$ or on aggregation. When the PRP (platelet rich plasma) was incubated in high glucose at 37^0C for 24 hrs, several differences were observed when compared to platelets incubated in 5 mM Glucose. PRP with EDTA as the anticoagulant, was divided into 3 groups : control (5 mM D-glucose medium) ; high mannitol (5mM D-Glucose + 40 mM Mannitol, as an osmotic control) and a high glucose group (45mM D-Glucose). In the high glucose group, compared to the mannitol and control groups, the first peak and second phase of thrombin-induced $[Ca^{2+}]_i$ were increased. The enhanced second phase was inhibited by CBDMB. In addition, the collagen-stimulated $[Ca^{2+}]_i$ response and aggregation were increased in the high glucose group. These results suggest that prolonged hyperglycemia in vitro can mimic platelet calcium abnormality and hyperfunction seen in diabetes, suggesting further that hyperglycemia may be the factor responsible for the platelet hyperactivity.

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LIST OF ABBREVIATIONS

AC	adenylate cyclase
BCECF	2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein
BCECF-AM	2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein acetoxy methyl ester
CBDMB	5-(4-chlorobenzyl)-2',4'-dimethylbenzamil
$[Ca^{2+}]_i$	cytoplasmic free calcium concentration
CP	creatine phosphate
CPK	creatine phosphokinase
CPA	cyclopiazonic acid
DAG	diacylglycerol
DCB	3',4'-dichlorobenzamil
DMSO	dimethyl sulfoxide
DTS	dense tubular system
EGTA	ethylene glycol tetraacetic acid

EIPA	5-(N-ethyl-N-isopropyl)-amiloride
G-6-P	glucose-6-phosphate
HEPES	6-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IDDM	insulin-dependent diabetes mellitus
IP_3	inositol triphosphate
$[Na^+]_i$	cytosolic free Na^+ concentration
NIDDM	noninsulin-dependent diabetes mellitus
NMDG	N-methyl-D-glutamine
PAF	platelet-activating factor
$[pH]_i$	cytosolic pH
PIP_2	phosphotidylinositol biphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PDGF	platelet-derived growth factor
PMCA	plasma membrane Ca^{2+}-ATPase
PRP	platelet rich plasma

ROC	receptor-operated channel
SR	sarcoplasmic reticulum
TXA_2	thromboxane A_2
VOC	voltage-operated channel
VSMC	vascular smooth muscle cell

INTRODUCTION

PREAMBLE

VASCULAR COMPLICATIONS OF DIABETES MELLITUS

Diabetes mellitus is a very common disease in North America. It is a complex metabolic derangement due to either relative or absolute insulin deficiency. Diabetes comprises a heterogeneous group of disorders characterized by high blood glucose levels and accompanied by characteristic long-term complications.

Four major types of diabetes have been defined: insulin-dependent diabetes mellitus (IDDM), non-insulin-dependent diabetes mellitus (NIDDM), gestational diabetes mellitus (GDM), and diabetes secondary to other conditions.

IDDM usually develops during childhood. The patients have prominent diabetes symptoms and extreme hyperglycemia. The insulin levels are substantially reduced. As a result, body fat is metabolized as a source of energy, and ketone bodies (acetoacetic acid and beta-hydroxybutyric acid) are released into the blood. Therefore, systemic metabolic acidosis is common in IDDM.

NIDDM usually develops in the adult. Genetic influences are key factors in the occurrence of type II diabetes. The patients have either normal or only slightly reduced amounts of insulin. They have hyperglycemia, but their cells are relatively deficient in insulin response (insulin resistance), so despite normal or elevated levels of insulin in the blood, the cells metabolize glucose insufficiently.

After the introduction of insulin therapy, the most common cause of morbidity and mortality in diabetic patients is mainly determined by the vascular complications (Brand et al., 1989; Singer et al., 1989; Sprafka et al., 1991). More than 75% of diabetic patients die from vascular diseases (Diabetes Epidemiology Research International Mortality Study Group, 1991; Songer et al., 1992, Morrish et al., 1990).

The vascular diseases in diabetes can be divided into two main categories. The first category is the microvascular disease affecting the smallest blood vessels, the capillaries, and the precapillary arterioles. These are specific to diabetes and are called microangiopathy, including retinopathy, nephropathy, and neuropathy. Diabetic retinopathy is one of the leading causes of blindness and visual impairment worldwide. Diabetic nephropathy is associated with a greatly increased risk of renal failure and death.

The second category of diabetic vascular diseases is large vessel disease affecting cardiac, cerebral, and peripheral vessels. These are called macroangiopathy, which is essentially an accelerated form of atherosclerosis. The macrovascular atherosclerotic disease causes serious morbidity and the largest fraction of excess mortality among people

with diabetes (Nathan, 1993). Patients with all forms of diabetes of sufficient duration are vulnerable to these complications.

Considering these consequences of diabetes it is very important to understand the pathogenesis of these diabetic vascular complications. To date, many pathological hypotheses have been developed to explain why there is an increased incidence, type, and prognosis of vascular diseases in diabetes mellitus. One pathogenic mechanism that has been suggested is that platelets from diabetics are hyperactive which contributes to the development of the accelerated atherosclerosis and diabetic microangiopathy (Colwell and Winocour, 1983; Pyorla et al, 1987, Ishii et al., 1992) (The evidence for this hypothesis will be reviewed in detail in a later section of this thesis). However why the platelets are hyperactive in diabetes is not clear.

Therefore, the overall objective of this project was to determine the mechanisms for platelet hyperactivity in diabetes mellitus. Before going into the research details, it is essential to have an overview of platelets and their involvement in diabetic vascular diseases.

PART I. GENERAL REVIEW OF PLATELETS

I.1 STRUCTURE

Platelets are very small anuclear cells (3um in diameter and 1 um thick). They were first observed in the middle of the 19th century but they were not firmly accepted as cellular constituents of blood until this century.

Platelets are formed from megakaryocytes, which are derived from haematopoietic, pluripotent stem cells (Levin and Evatt, 1979). Platelets are produced in the bone marrow by the pinching off parts of megakaryocyte pseudopodia and by mechanical fragmentation of larger portions of megakaryocyte cytoplasm during passage through the microcirculation of the lung (Pennington, 1981). Each mature megakaryocyte can form about 4,000 platelets. The eventual number of platelets released is dependent on the final platelet number reached. In humans differentiation of the stem cell into platelets takes about 10 days (Hoffbrand and Pettit, 1984). The production of platelets from megakaryocytes is controlled by a humoral factor, thrombopoietin (McDonald, 1974).

The normal platelet count in humans is approximately $2.5X10^5$/ml whole blood (Harker and Slichter, 1972), and their half-life in the circulation is about 8-11 days (Aster, 1969). Platelets can be removed from the circulation either through the involvement in a haemostatic plug in response to vascular injury or by the reticuloendothelial system (Aster, 1969).

Platelets in circulating blood or in anticoagulated samples of platelet-rich plasma are disc-shaped. Their structure can not be seen clearly by light microscope, but electron microscopy has revealed a well-defined and complex structure, which is consistent with their active role (White, 1972; 1974; 1979; White and Krivit, 1967).

The outermost layers of platelets are comprised of a glycocalyx and a plasma membrane. The plasma membrane is rich in glycoproteins and is invaginated to form the surface-connected or open canalicular system (OCS). The OCS is very close to the dense tubular system (DTS) which is derived from the smooth endoplasmic reticulum of megakaryocytes and is similar to the endoplasmic reticulum of other cells (Streb et al., 1983; Brass and Joseph, 1985; Ross et al., 1989). When secretion occurs, the OCS can greatly increase the surface area of platelets (Escolar and White, 1991). The DTS contains a Ca^{2+}-ATPase, so it is the most likely internal organelle for Ca^{2+}-sequestration in platelets (Behnke, 1970; Manashi et al., 1984; Adunyah and Dean, 1985; Enyedi et al., 1986).

Immediately below the plasma membrane is a ring of microtubules that run around the edge of the disc and maintain the discoid architecture of resting platelets (White and Krivit, 1967). Platelets also contain submembrane microfilaments, including alpha-actin and actin-binding protein (Davies, 1984; Siess, 1989) which are involved in pseudopodia formation upon platelet activation. Platelets lack nuclei and any significant components of protein synthesis, but they contain other major components common to larger cell

types. The intracellular organelles in platelets include: alpha- and dense granules, peroxisomes, lysosomes, mitochondria and discrete particles of glycogen.

I.2 FUNCTION

The major physiological function for circulating platelets is in haemostasis. When vascular injury occurs, platelets rapidly adhere to exposed subendothelium (particularly collagen) and induce the release of their granule contents such as ADP, serotonin and the synthesis and release of thromboxane. These substances then recruit more circulating platelets to the site of injury and the platelets clump together to form a hemostatic plug. Platelet activation also promotes the coagulation cascade resulting in the generation of thrombin. Thrombin can act as a powerful platelet activator and thus, amplifies the haemostatic process. In addition, thrombin can catalyse the formation of fibrin which consolidates the plug. Therefore this platelet plug temporarily seals off the damaged vessel wall. This process is called haemostasis.

Platelets can be activated by many physiological (thrombin, collagen, ADP, adrenaline, vasopressin, serotonin) and non-physiological substances (divalent cationophores, phorbol esters). In addition, platelets can be partially activated when brought into close contact with foreign surfaces (e.g. glass) as well as with each other (close cell contact). After activation the platelet responses can be classified into 2 categories: one is reversible responses including shape change, adhesion and primary (reversible) aggregation, and the other is secondary (irreversible) aggregation (Siess, 1989). The reversible responses are used to repair small defects in the subendothelium

and to close gaps in the endothelial lining (Tranzer and Baumgartner, 1967; Baumgartner, 1972). The irreversible responses are predominantly involved in the formation of a haemostatic plug.

Shape change

One of the first physiological responses of platelets upon stimulation is shape change. Resting platelets are discoid with a smooth, rippled surface. When platelets are activated, they lose their discoid shape, become spherical and extrude pseudopodia which is observed as a small decrease in light transmission (White, 1974). This change in shape is associated with constriction of the ring of microtubules running around the circumference of the platelet and centralization of the organelles within the microtubule ring. Shape change involves an increase in the surface area of the platelet membranes, and most probably results from the evagination of the surface-connected canalicular system (White, 1974). Shape change is not an essential prerequisite for aggregation. Extracellular cations (Ca^{2+}, Mg^{2+}) are not required for platelet shape change, unlike the subsequent reaction of aggregation.

Most platelet stimuli (except adrenaline) can induce full platelet shape change (White, 1974; Affolter and Pletscher, 1982). Although most platelet agonists induce the response with $[Ca^{2+}]_i$ well below the threshold level, an increase in cytosolic calcium alone can produce full platelet shape change (Rink et al., 1982; Hallam and Rink, 1985)

Adhesion

Adhesion can be induced by vascular injury which causes gaps to open in the endothelium. After the exposure of subendothelium, platelets rapidly adhere to the sub-endothelial connective tissue (Tranzer and Baumgartner, 1967) by interacting with 2 proteins: collagen and von Willebrand Factor (vWF).

vWF is a complex, multimeric glycoprotein (a large adhesive glycoprotein) participating in a variety of interactions aiding in the formation of a platelet plug following vascular injury. It is synthesized by endothelial cells, megakaryocytes and is also found in platelets. vWF has at least two essential functions in hemostasis. First, vWF interacts with the GP Ib/IX/V receptor complex on the platelet surface and is required for the adhesion of platelets to the subendothelium in areas of high shear, such as in small arteries or in the microcirculation. Secondly, it is a component of the main fraction of the blood clotting factor VIII molecule and stabilizes the factor VIII in circulation (Tschopp et al., 1974; Jaffe et al., 1974).

Collagen fibrils are required for platelet-stimulating activity and collagen acts both as a solid to which platelets adhere and as a primary agonist for platelet aggregation. (Shadle and Barondes, 1982; Santoro,1986). Definite reactivity has been shown for collagen types I and III which are located in the deeper layers of vessel walls and in atherosclerotic plaques. Little adhesion was found to collagen type IV and only a very limited interaction was found with collagen type V which are in the subendothelium (Packham and Mustard, 1984).

Secretion

Platelets contain 3 types of storage granules: dense granules, alpha-granules and lysosomes. On proper stimulation of the platelets, the contents of the storage organelles are specifically extruded to the cell's environment without loss of constituents from other subcellular compartments. This specific extrusion occurs by exocytosis and is called platelet secretion. The process takes 10 to 120 seconds, depending on the strength of the stimulus and which secreted substance is measured. Many of the secreted substances have either direct actions on cells, including the platelets themselves, or are converted to physiologically active substances in plasma or on cell surfaces.

Alpha-granules and dense granules contain paracrine chemical messengers that amplify the original platelet stimulus by a cascade of positive feedback. Dense granules release ADP, ATP, Ca^{2+} and serotonin. Alpha-granules secrete coagulation factors such as fibrinogen and vWF; platelet-specific proteins such as beta-thromboglobulin and platelet factor 4 as well as growth factors such as platelet-derived growth factor (PDGF) (Kaplan et al., 1979; Holt and Niewiarowski, 1985). Lysosomal granule contents are released only after strong agonists such as thrombin and high concentration of collagen.

In addition, activated platelets can release active substances such as PGs (PGG_2 and H_2), thromboxane A_2 (Marcus, 1978) and platelet activating factor (PAF) (Chignard et al., 1979). These substances are newly synthesized rather than stored and they are used to recruit further platelets to the site of vascular injury.

Aggregation

Platelet aggregation is the process by which platelets interact with one another to form a hemostatic plug or thrombus. It is the critical step in platelet reactions because it permits mechanical plugs to be formed rapidly at the site of vascular injury and ensures effective haemostasis.

In vivo, the primary plug is very unstable and requires reinforcement with proteins derived from the clotting cascade such as fibrinogen. Binding of fibrinogen to platelets is essential in platelet aggregation (Peerschke, 1985). It represents the final common pathway for platelet aggregation regardless of the mechanisms of activation. Fibrinogen is a dimeric glycoprotein and its structure is suited for acting as a molecular bridge between platelets. The receptor for fibrinogen on platelets has been identified as the membrane integrin glycoprotein complex IIb/IIIa (GpIIb/IIIa) (Kunicki et al., 1981; Newman et al., 1982). In resting conditions, the GPIIb/IIIa is unavailable for binding, even in the presence of plasma fibrinogen and Ca^{2+}. The fibrinogen receptor must first be exposed by platelet activators such as ADP, thrombin, collagen and adrenaline. The exact mechanism of fibrinogen receptor exposure is not clear , but it may involve a conformational change in glycoproteins IIb and IIIa such that they form a high affinity receptor for fibrinogen (Phillips et al., 1991). External Ca^{2+} is a cofactor in the formation of fibrinogen-platelet aggregates (Shattil et al., 1985). When bound to platelets, fibrinogen links two or more platelets together by forming molecular bridges. In addition to interacting with fibrinogen, the protein binding sites on GPIIb/IIIa can also accommodate other adhesive molecules such as vWF, fibronectin and vitronectin.

In vitro, 2 types of aggregation can be identified: a primary aggregation, which is reversible and occurs without the release reaction. The second is a secondary aggregation, which is irreversible and occurs with platelet secretion.

The primary (reversible) aggregation can be induced by low concentrations of platelet agonists in the presence of extracellular Ca^{2+} and is associated with fibrinogen binding to its receptor (Siess, 1989).

The secondary (irreversible) aggregation depends on the ability of platelets to release paracrine chemical messengers. The released substances include TXA_2 which is derived from arachidonic acid (AA) (Hamberg et al., 1975), ADP and Ca^{2+} which are secreted from dense granules (Holmsen et al., 1969) and adhesive proteins from alpha-granules (Kaplan et al., 1979). These messengers amplify the original stimulus, to recruit additional platelets from the circulation. They can also provide localized, high concentrations of fibrinogen, fibronectin, thrombospondin and vWF (alpha-granule secretions) at the platelet surface, which can aid platelet adhesion to exposed sub-endothelial collagen and strengthens platelet-platelet interactions within the developing thrombus. This self-amplification process ensures an irreversible platelet mass that is large enough to plug the area of vascular damage.

I.3 Signal Transduction Mechanisms

Platelet activation is initiated by the binding of agonists to their specific receptors on the platelet membrane. The signal is then transferred to the second messengers by a

family of signal transducing GTP binding proteins (G-proteins). Second messengers such as calcium then act on the target proteins producing functional responses.

G-proteins

G proteins are a closely related family of GTP-binding proteins that mediate the interaction between cell-surface receptors and intracellular effectors such as enzymes and ion channels. A total of seven G proteins and 3 different target enzymes have been described in platelets.

Phospholipase C

In platelets, 4 agonists are believed to cause G-protein-dependent activation of the phospholipase C (PLC) pathway: thrombin, vasopressin, PAF, and thromboxane endoperoxide analogues such as U46619 or U44069. There are two G proteins that mediate this pathway. One is Gp, which interacts with receptors for thrombin, PAF, and vassopressin and is pertussis toxin-sensitive. The other, Gp' interacts with thromboxane receptors and is pertussis toxin-insensitive.

After activation, PLC cleaves phosphatidyl inositol-bisphosphate (PIP_2) forming 2 second messengers: inositol trisphosphate (IP_3) and diacylglycerol (DAG). The water soluble IP_3 mobilizes calcium ions from the dense tubular system, via binding to IP_3 receptors and the concentration of cytosolic Ca^{2+} is increased. DAG remains in the plasma membrane and activates a key enzyme in platelet activation, protein kinase C (PKC).

PKC is a member of a ubiquitous family of enzymes that phosphorylates serine and

threonine residues of intracellular proteins involved in signal transduction (Nishizuka, 1988). It was first found as a Ca^{2+} and phospholipid-dependent protein kinase. Now it has been estabilished that PKC is not a single molecular entity but is a family of a few closely related isozymes. Untill now, 12 PKC isoforms have been identified, as reviewed by Puceat and Vassort (1996). They are divided into 3 groups: (1) Conventional (alpha-, beta 1 and 2, gamma): these isoforms contain a Ca^{2+}-binding site (C_2 region) and require Ca^{2+} for activation. (2) Novel (delta-, epsilon-, theta-, eta-, and mu): these are Ca^{2+}-insensitive PKCs. The isoforms in these 2 groups have 2 cysteine-rich domains (C_1 region) which bind phorbol esters and can be activated by diacylglycerol and phorbol esters. (3). Atypical (zeta, lambda and tau). They are called atypical because these isoforms lack both C_2 and one cysteine-rich domain and are therefore Ca^{2+}-insensitive and cannot be activated by DAG and phorbol esters. It has been found that human platelets mainly contain PKC alpha, beta 1 and 2, delta, and zeta isoforms (Baldassare et al., 1992).

In the resting state, most of the PKC isoforms are located in the cytosol. After the G-protein-mediated activation of PLC and production of IP3 and intracellular calcium release, the calcium binds to the cytosolic PKC and exposes the phospholipid binding site. This binding of calcium translocates PKC to the membrane where it interacts with DAG and transforms into a fully active enzyme. The increase in cytosolic Ca^{2+} together with several phosphorylation processes mediated by PKC triggers the different platelet functions.

Phospholipase A_2

It has been shown thrombin-induced release of ^{3}H-arachidonate from permeabilized platelets was GTP dependent and was inhibited by pertussis toxin (Kajiyama et al., 1989) suggesting PLA_2 is G-protein linked. However this G protein has not been identified. PLA_2 plays a role in platelet activation by most agonists but is particularly critical for agonists such as epinephrine that do not activate PLC. Cytosolic calcium and pH can modulate the activity of PLA_2.

Activation of PLA_2 cleaves the sn-2 acyl bond of phospholipids (phosphatidylcholine and phosphatidylethanolamine) at the cytoplasmic site of the plasma membrane and liberates arachidonic acid. Arachidonic acid is subsequently converted to prostaglandin endoperoxides (PGG_2 and PGH_2) and thromboxane A_2 (TXA_2) via the respective catalysis by cyclooxygenase and thromboxane-synthase which are located in the dense tubular system. TXA_2 further augments platelet activation via binding to specific receptors coupled to PLC.

Adenyl Cyclase

Activation of adenyl cyclase (AC) by platelet inhibitors triggers the synthesis of cAMP via a stimulatory G protein, Gs. An increase in cAMP inhibits different steps in the activation pathways, such as the liberation of arachidonic acid, the formation of IP_3, and the mobilization of calcium ions. Certain agonists activate receptors that prevent the rise in cAMP via an inhibitory G protein, G_i. A decrease in cAMP below the level of resting platelets does not induce platelet functions; instead it is thought to enhance signal

generation via PLA_2 and PLC, thereby inducing synergistic activation between different stimuli.

Calcium

The fundamental role of calcium in cell signaling was recognized a long time ago by Ringer in classic experiments on the role of calcium in muscle contraction (Ringer, 1882) and by a number of striking findings concerning motility and nervous activity. Similar to most cells, cytosolic calcium is a key second messenger in platelet function.

Many processes in platelets are Ca^{2+}-dependent such as adhesion, shape change, aggregation, secretion, Ca^{2+} transport by Ca^{2+} or Mg^{2+} ATPase, phosphorylation of myosin light chain, activation of phospholipase A and C and activation of cyclooxygenase. Calmodulin, the Ca^{2+}-binding protein, is the mediator of some Ca^{2+}-regulated processes.

The total concentration of Ca^{2+} in platelets is in the mM range. In resting state, most of the Ca^{2+} is concentrated in storage sites such as the plasma membrane, dense tubular system, mitochondria, storage granules and possibly other locations. The resting cytosolic concentration of ionized Ca^{2+} is about 100 nM or less. After stimulation, the progressive increase in the free cytosolic Ca^{2+} concentration reflects the graded response of platelets to stimuli of varying intensity. For example, most agonists can induce aggregation with a rise in $[Ca^{2+}]_i$. If the $[Ca^{2+}]_i$ rise is large (>1uM), $[Ca^{2+}]_i$ rise alone can induce platelet aggregation (Rink et al., 1982).

On the other hand, some aspects of platelet activation are "Ca^{2+}-independent". For example, there is a Ca^{2+}-independent pathway for platelet aggregation. It was reported that collagen evokes aggregation without a detectable rise in $[Ca^{2+}]_i$ in platelets exposed to aspirin (Watson et al., 1985), although it is still controversial as to whether collagen produces a $[Ca^{2+}]_i$ increase. The mechanism of this aggregation can be attributed to diacylglycerol which is a protein kinase C activator and which in isolation can produce aggregation (Rink et al., 1983).

However this "Ca^{2+}-independent" conclusion is contentious because $[Ca^{2+}]_i$ elevation can be observed with aequorin in platelet activation induced by collagen even in the presence of aspirin as well as in platelet activation induced by phorbol ester and diacylglycerol (Ware et al., 1986). The commonly used Ca^{2+} sensitive dyes such as fura-2, is an averaging technique and may not detect small local zones of increased $[Ca^{2+}]_i$.

I.4 PROTEIN PHOSPHORYLATION

Platelet functional responses involve the phosphorylation of many proteins. The profile of protein-phosphorylation is dependent upon the concentration and agonist used.

Ca^{2+} is important in the regulation of platelet protein phosphorylation. It has been shown that A23187, the Ca^{2+}-ionophore, can evoke ^{32}P incorporation into a number of platelet proteins (Lapetina et al., 1986). Proteins that become phosphorylated in a Ca^{2+}-dependent fashion include the 20-KDa myosin light chain. This is achieved by a specific Ca^{2+} /calmodulin-dependent protein kinase, myosin light chain kinase (MLCK). Myosin

phosphorylation results in the association of myosin with the cytoskeleton of the platelets involved in shape change and secretion.

Pleckstrin, a 47K protein is the major substrate for protein kinase C. Its function is unclear but may be related to the initiation of storage granule secretion.

1.5 Individual Agonists

Many agonists (thrombin, collagen, ADP, thromboxane A2, and epinephrine) can activate platelets. Collagen and thrombin are primary activators at sites of vascular injury, whereas ADP and TxA_2 are released from platelets and help to recruit additional platelets into a growing platelet plug. The platelet surface contains receptors which specifically bind to these agonists and to cryptic receptors (such as the fibrinogen receptor) (Marguerie et al., 1986) which become available upon platelet activation.

Table 1. Aggregating agents and other substances that bind to platelets

Aggregating Agents	Receptor
Thrombin	GPIb-alpha
	7 transmembrane domain receptor
ADP	Aggregin?, 100 kDa
Collagen	GP Ia/IIa, GPIV (CD36), GPVI
Thromboxane A_2	PGH_2/ TXA_2 receptor
Platelet-activating factor	PAF receptor
Serotonin	S_2-subtype (5-HT_2 receptor)
Epinephrine	alpha-2 adrenergic receptor

Vasopressin	V_1-type
Fibrinogen	GPIIb/IIIa
von Willebrand factor	GPIb/IX/V, GPIIb/IIIa
Fibronectin	GPIIb/IIIa
Thrombospondin	?GPIV?

Note: From Hourani and Cusack 1991; Siess 1989

ADP

ADP was the first compound shown to aggregate platelets in vitro (Born, 1962). It is an exclusively intracellular substance and appears in plasma only through lysis of tissue or blood cells and by dense granule secretion in platelets. ADP is a weak platelet agonist and it can cause rapid shape change, dense granule and alpha-granule secretion, exposure of fibrinogen binding sites and platelet aggregation.

The platelet ADP receptor belongs to the P_2 class of purinergic receptors, whereas the P_1 class recognizes adenosine (Burnstock, 1978). P_2 receptors can be further classified into P_{2x} (mediating contraction of smooth muscle) and P_{2y} (mediating relaxation) based on agonist potencies. The platelet ADP receptor resembles more closely the P_{2y} than the P_{2x}, however, it has many different properties from the P_{2y} receptor because it demonstrates much more rigid structural requirements for agonists than conventional P_{2y} receptors in other tissues (Gordon, 1986). In addition, ATP is a competitive antagonist of the platelet ADP receptor (Macfarlane and Mills, 1975), whereas there are no true

competitive antagonists of the P_{2x} or P_{2y} receptors (Burnstock, 1978). Therefore the platelet ADP receptor was assigned another name: P_{2T} (Gordon, 1986). At present, the platelet ADP receptor is not identical to any other receptors for adenine nucleotides (Hourani and Cusak, 1991). It appears to be unique and has not been found in any other tissues.

Despite the important role in the activation of platelets, the ADP receptor on the platelet membrane has not been completely characterized. It was reported that a 100-KDa protein (aggregin) may be the ADP receptor (Colman et al, 1988). The signal transduction mechanisms after ADP binding have not been elucidated. It has been shown that ADP can activate a receptor operated channel (ROC) that may provide an important mechanism for ADP to rapidly activate human platelets (Mahaut-Smith et al., 1990). The G proteins with which the putative ADP receptor interacts have not been established. During ADP-induced aggregation, phospholipase C has not been shown to be activated and IP_3 formation does not occur (Packham et al., 1993).

Collagen

Collagen is an important adhesion molecule and primary agonist in platelet aggregation during haemostasis. Released ADP and TXA_2 contribute to collagen-induced aggregation and secretion since it has been shown that the responses can be strongly inhibited by aspirin and by enzymatic conversion of secreted ADP to inactive adenine nucleotides (Kinlough-Rathbone et al., 1977).

The receptor for collagen on platelets has not been clearly illustrated. There are a few candidates for the collagen receptor in platelets: glycoprotein complex GPIa/IIa (Coller, 1989), glycoprotein IV (CD36) (Tandon et al., 1989), and glycoprotein VI (Moroi et al., 1989). Patients with a deficiency of GPIa/IIa have impaired hemostasis, but individuals whose platelets do not express GPIV do not have hematological problems.

The signal transduction pathway for collagen is not known. Two different general mechanisms have been described. One is a G-protein mediated mechanism, however this seems improbable since none of the glycoproteins which are thought to be collagen receptors have the 7-transmembrane domain architecture associated with G protein-coupled receptors.

Another mechanism is the tyrosine kinase pathway (Shattil and Brugge, 1991). It has been proposed that after collagen stimulation, tyrosine kinase is activated, phospholipase $C_{\gamma 2}$ isoform is phosphorylated and IP_3 is produced. This mechanism is possible since there is evidence that collagen-induced inositol phosphate formation in platelets is blocked by staurosporine, a nonselective inhibitor of tyrosine and serine/threonine kinases (Blake et al., 1993).

Although collagen induces formation of inositol phosphates in platelets (Watson et al., 1985), it is still controversial whether this is associated with elevation of cytosolic Ca^{2+}. On one hand, it has been shown that in the presence of cyclo-oxygenase inhibitors, collagen (20ug/mL) does not induce a measurable rise in cytosolic Ca^{2+} in platelets loaded with fura-2 or quin-2 (Rink et al., 1983; Watson et al., 1985; Pollock et al., 1986), despite

the fact that these agents are able to induce activation of the phosphoinositide pathway. On the other hand, it was observed that collagen (50ug/mL) can induce a rise in cytosolic Ca^{2+} in the presence of a thromboxane receptor antagonist, an ADP-scavenger system and a fibrinogen antagonist (Smith et al., 1992). Recently, Poole and Watson (1995) reported that collagen (100ug/mL) can by itself induce a rise in cytosolic Ca^{2+} in single human platelets through a tyrosine kinase-mediated pathway .

Epinephrine

Epinephrine and norepinephrine are released into the circulation from the adrenal medulla during stressful situations. Platelets have predominantly alpha-2 adrenergic receptors. The signal transduction occurs by activating G_i, thus inhibiting adenyl cyclase and producing a decrease in cAMP. Catecholamines are not complete agonists and a supraphysiologic concentration of epinephrine is required for platelet aggregation. Thus in vivo, catecholamine activation involves cooperative effects (Cryer, 1980). Catecholamines are capable of potentiating the effects of other agonists, such as thrombin, ADP, and collagen.

Thrombin

Thrombin is formed from prothrombin in plasma during the coagulation process. Prothrombin is synthesized in the liver and thrombin is a proteolytic product of prothrombin. When platelets are stimulated, a transbilayer movement of membrane phospholipids brings procoagulant phospholipids to the surface of the platelets. On the

activated platelet surface 2 steps of the intrinsic coagulation pathway are greatly accelerated, leading to the formation of thrombin (Mann et al., 1992). In addition, the extrinsic coagulation pathway makes a major contribution to thrombin formation when tissue factor is exposed at a site of injury.

Thrombin has 2 major roles in the development and stabilization of platelet aggregates. Firstly, thrombin is the most potent aggregating agent, and it can cause aggregation without any contribution from thromboxane A_2 or ADP, although these substances do become available during thrombin-induced platelet aggregation and augment the response. Secondly, thrombin converts fibrinogen to fibrin, which forms in and around platelet aggregates and stablizes them from disruption by the force of flowing blood. Besides its role in haemostasis, thrombin has other effects. For example, thrombin can stimulate cell replication (Chen and Buchanan, 1975) and modulate vascular contractility (White et al, 1980).

The thrombin receptor has been isolated by using an expression cloning approach from a human magakaryoblastic Dami cell line (Vu et al., 1991) and hamster fibroblasts (Rasmussen et al., 1991). Like other receptors which interact with G proteins, the thrombin receptor comprises a single polypeptide with 7 transmembrane domains and an extracellular N-terminus. The activation of the thrombin receptor is rather different from the classical receptor agonist interaction (Coughlin et al, 1992). Simple binding of thrombin to a receptor does not result in a signal that activates platelets. Thrombin activates its receptor through proteolytic cleavage of the receptor. The extracellular

extension of the thrombin receptor is cleaved by thrombin at a site between Arg41 and Ser42 (Vu et al., 1991), exposing a new NH_2-terminal and releasing a tethered receptor fragment. The newly exposed NH_2-terminal then binds to an unidentified region of the receptor and activates it. Peptides whose sequence corresponds to this tethered ligand can mimic many, if not all, of the effects of thrombin on platelets and other vascular cells. SFLLRN, a peptide of 6 amino acids, is a potent platelet agonist that induces the same platelet reactions of shape change, aggregation, secretion and thromboxane A_2 formation as are triggered by thrombin. It was also found that thrombin activity is Na^+-dependent (Wells and DiCera, 1992). Extracellular Na^+ is required for the enzymatic activity of thrombin. Shortly after activation, thrombin receptors become resistant to reactivation by either thrombin or peptides. This loss of function could be due to a combination of receptor desensitization, possibly caused by receptor phosphorylation, and receptor internalization.

Other investigators reported that human platelets have at least two types of thrombin receptors, with high and moderate affinity for thrombin (Greco and Jamieson, 1991; Seiler et al., 1991) and coupled to different platelet responses. Harmon and Jamieson (1986) also provided evidence for one class of receptor exhibiting both low and high affinities, each coupled to different platelet responses.

The first type (R_1) is mediated by a small number (50 sites/platelet) of high-affinity (K_d=0.3 nM) binding sites and low concentrations of thrombin (1nM, 0.1 NIH units/ml or less) are required. After this receptor is stimulated the activated pathways include

inhibition of stimulated adenylate cyclase, secretion of acid hydrolases, and activation of phospholipase A_2.

The second receptor (R_2) is mediated by an intermediate number of binding sites (1700/platelet) with moderate affinity (K_d=11 nM) and requires moderate concentrations of thrombin (2 nM, 0.2 NIH units/ml or more). This receptor is linked to phospholipase C (PLC) activation which convert PIP_2 to IP_3 and DAG. IP_3 mobilizes Ca^{2+} from DTS. Ca^{2+} entry from the external medium also occurs and the cytosolic Ca^{2+} concentration is increased (Rink and Sage, 1990). DAG stimulates protein kinase C, which phosphorylates pleckstrin, a 47-Kda protein. Other protein phosphorylations, such as tyrosine phosphorylation, have been shown to be involved. These responses result in platelet aggregation and exposure of fibrinogen binding sites.

Biochemically the identity of other thrombin receptors apart from the cloned thrombin receptor on human platelets has not been established. The high affinity receptor may be the glycoprotein Ib-alpha which has only one transmembrane domain (Roth, 1991). The cloned thrombin receptor is probably the moderate-affinity receptor with 7 transmembrane segments (Greco et al, 1992).

Inhibitory systems

In the human body there are systems to control blood coagulation at several points. One point is the endothelial cell which maintains normal blood fluidity and a nonthrombogenic surface.

Endothelial cells synthesize and release PGI_2, and nitric oxide (endothelium-derived relaxing factor) which inhibit platelet aggregation and adhesion. They inhibit platelet function by increases in intracellular cAMP and cGMP concentration, respectively.

Endothelial cells also constitutively synthesize thrombomodulin, an intrinsic membrane receptor (Emson, 1989). Thrombomodulin binds thrombin forming a thrombomodulin-thrombin complex which converts the protease into a potent activator of protein C. In the presence of protein S, activated protein C acts as an anticoagulant, inactivating factors Va and VIIIa. In this way it effectively removes their involvement in the formation of active enzyme-phospholipid complexes of the coagulation cascade. Therefore the activity of thrombin is regulated and confined to the site of vascular injury.

Endothelial cells also continually produce tissue plasminogen activator (t-PA) which initiates intravascular fibrinolysis at the vessel surface (Van Hinsbergh, 1988). Fibrinolytic activity is a limiting factor in the growth and dissolution of thrombi. t-PA is the enzyme in the fibrinolytic pathway that converts plasminogen to plasmin, and plasmin acts to degrade fibrin, fibrinogen, and factors V and VIII.

I.6 PATHOLOGY (INVOLVEMENT IN ATHEROSCLEROSIS)

Platelets are essential components in thrombosis formation. The arterial thrombi are mainly composed of platelets. These platelet-fibrin thrombi cause the thromboembolic complications of atherosclerosis: myocardial infarction, thrombotic strokes, and peripheral vascular disease. The processes involved in the formation of arterial thrombi are

essentially the same as those in the formation of hemostatic plugs, thus approaches to prevent arterial thrombosis have the risk of causing bleeding episodes.

Platelets are also involved in the pathogenesis of atherosclerosis. Atherosclerosis is a focal intimal disease of arteries within a range of size from the aorta down to approximately 3 mm external diameter. For example, the aorta, coronary, cerebral, internal carotid, iliac, femoral and mesenteric arteries are common sites of atherosclerotic lesions. The primary pathological characteristic is the presence of focal thickenings (plaques) of the innermost layer (intima) of elastic and muscular arteries. The lesions tend to form at the branch points of arterial vessels. There are 3 typical pathological lesions:

Pathology of atherosclerosis

The fatty streak: The ubiquitous fatty streak is the first recognizable change of atherosclerosis and appears in the intima and inner media of affected arteries. It is commonly found in children. These lesions are fatty yellowish small dots or streaks barely raised above the intimal surface. At the microscopic level, each fatty streak is made up of a focal collection of lipid-filled cells (foam cells) within the intima. The foam cells are mostly derived from macrophages although some smooth muscle cells which contain lipid are often present as well.

The fibrous plaque: Fibrous plaques are the hallmark of atherosclerosis. These may cause narrowing of the artery, predispose to thrombosis and may calcify. They are rounded, raised lesions, usually off-white to white in color superficially, and perhaps a

centimeter or more in diameter. A typical fibrous plaque consists of (a) a fibrous cap, which is composed mostly of smooth muscle cells, a few leukocytes and relatively dense connective tissue (elastin, collagen fibrils, proteoglycans, and basement membrane). (b) a cellular area which is beneath and to the side of the cap. The main composition of this area is a mixture of macrophages, smooth muscle cells, and T lymphocytes; and (c) a deeper "necrotic core" which contains cellular debris, extracellular lipid droplets, cholesterol crystals and calcium deposits. The necrotic core often contains many large foam cells originating from both macrophage and smooth muscle cells.

It is controversial whether the childhood fatty streaks are related to adult fibrous plaques. There is evidence that fibrous plaques are derived from fatty streaks. However, not all fatty streaks necessarily progress into advanced atherosclerotic plaques. The aorta and coronary arteries of adults show all stages of plaque development, implying that new lesions are generated throughout adult life.

The complex lesion: The complex lesion is the most common type of atherosclerotic lesion that produces significant circulatory change and clinical disease. It develops from preexisting fibrous plaques as a result of one or a combination of several pathological changes such as calcification, ulceration, thrombosis with deposition of fibrin and platelets, and hemorrhage.

Pathogenesis of atherosclerosis

There are many hypotheses concerning the pathogenesis of atherosclerosis, including the response to injury hypothesis, the monoclonal hypothesis, the lipid infiltration hypothesis and the inflammatory hypothesis. Different hypotheses emphasize the role of different cells. The cells involved include inflammatory cells (monocytes/macrophages), arterial smooth muscle cells, endothelial cells and platelets. Lipids and lipoproteins also play an important role. In this section, only the two hypotheses which emphasize platelets will be reviewed in detail.

It is well known that platelets play important roles in the pathogenesis of atherosclerosis (Ross , and Glomset , 1976; Ross, 1986; Sinzinger, 1986). Platelets can contribute to atherosclerosis by forming mural thrombi and by secreting mitogenic factors such as platelet-derived growth factor, which stimulate the proliferation of cells in the vessel wall.

There is strong evidence supporting the role of platelets in atherosclerosis. It has been shown that pigs with homozygous von Willebrand's disease are resistant to thrombosis (Fuster and Griggs, 1986) and to the development of spontaneous atherosclerosis (Fuster et al, 1982). Pigs fed a high-cholesterol diet but affected with severe von Willebrand's disease, only developed intimal infiltrates of lipid in their aortas, whereas the control animals without vWF disease received the same diet and showed extensive proliferative lesions of the VSMC (Fuster et al., 1978). If platelets are absent or less abundant at sites of endothelial injury induced by an indwelling catheter (Moore et

al., 1976) or in case of de-endothelialization caused by a balloon catheter (Cohen and McComb, 1968; Friedman et al., 1977) or if platelet function is pharmacologically inhibited by dipyridamole in experimentally induced homocysteinuria (Harker et al, 1976), then the VSMC intimal migration and proliferation that usually accompany such injury will not occur.

Response-to-injury hypothesis : This hypothesis was formulated by Ross and Glomset in 1976. It proposed that "injury"to the endothelium is the initiating event in atherogenesis (Ross and Glomset, 1976). Chronic minimal injury to the arterial endothelium is caused mainly by a disturbance in the pattern of blood flow in certain parts of the arterial tree, such as bending points and areas near branching vessels. In experiments in animals, chronic mild endothelial injury may also be potentiated by hypercholesterolemia, circulating vasoactive amines, immunocomplexes, infection and chemical irritants in tobacco smoke.

When there is injury or loss of the endothelium, platelets adhere rapidly to the exposed subendothelium such as collagen, which leads to the release of granule contents from adherent platelets. These include PDGF, ADP and serotonin. ADP and serotonin can cause platelet aggregation thus recruiting other platelets to the site of injury. In addition, the arachidonate pathway is activated and TXA_2 is generated and released which can then causes platelet aggregation and release of granule contents from platelets not directly adherent to the collagen. Under some conditions, such as disturbed flow, activation of coagulation occurs at the site of vessel injury and thrombin may be

generated, which can cause further platelet aggregation and release. Thrombin can also convert fibrinogen to fibrin, which stablizes the platelet aggregates. Thus, a mural thrombus is formed. If the thrombus persists and becomes organized, it can become incorporated into the vessel wall and would thereby further contribute to the atherosclerotic process. Alternatively, these thrombi may occlude an already narrowed vessel or they may break away to lodge elsewhere.

In response to the mitogenic and chemotactic factors (i.e. PDGF) from the platelets and other cells in the vessel wall, smooth muscle cells would migrate into the intima where they would proliferate and secrete connective tissue components.

Therefore, if there is vessel injury, platelets make major early contributions to the development of atherosclerosis and its thromboembolic complications. However this hypothesis cannot explain some atherosclerosis such as in cases of hypercholesterolemia in which there is no evidence of loss of endothelium. This leads to the "revised response-to-injury hypothesis" or "lipid infiltration hypothesis".

Lipid infiltration hypothesis : This hypothesis is centered around altered plasma lipids and hypercholesterolemia, and involves the formation of extensive lipid-rich lesions without evidence of loss of endothelium or thrombosis.

Vascular injury or damage can be divided into three types (representing stages of increasing severity): Type I, functional alterations of endothelial cells without substantial morphologic changes; Type II, endothelial denudation and intimal damage with intact

internal elastic lamina; and Type III, endothelial denudation with damage to both the intima and media.

In hypercholesterolemia, there is only type I injury. In this condition, adherence of monocytes to a modified, but intact, endothelium is one of the earliest events observed (Ross 1986, 1993). These monocytes then migrate into the vessel wall and accumulate lipid to form macrophage foam cells. In response to mitogens produced by macrophages and other cells in the vessel wall smooth muscle cells also migrate from the media into the intima and proliferate. In addition, they secrete collagen, proteoglycans, and other connective tissue components. Eventually, as a result of the build up of cellular and matrix material in the vessel wall, there is disruption of the underlying endothelium, exposing the underlying tissue. Then, platelets can adhere and release their granule contents. These released materials include growth factors which can cause further atherogenic changes in the vessel wall. LDL and VLDL may deliver cholesterol to the injury site.

Although these 2 hypotheses differ in their initiating factors,there are some similarities. For example, both emphasize that smooth muscle cell migration and proliferation are key events in atherosclerosis (Moore, 1985; Mustard et al., 1988; Ross 1986, 1993). If vessel injury and hypercholesterolemia occur together, the effects on the development of atherosclerosis are probably additive or even synergistic (Moore, 1989). Interestingly activated platelets also have cytotoxic effects on endothelial cells, which may

cause plasma infiltration of blood cells and lipids (Numano and Kishi, 1995). This finding suggests that platelets play important roles in the initiation of atherosclerosis.

PART II. PLATELETS AND DIABETIC VASCULAR COMPLICATIONS

II.1 EVIDENCE OF PLATELET INVOLVEMENT IN DIABETIC VASCULAR COMPLICATIONS

Platelets are involved in the pathogenesis of atherosclerosis in the nondiabetic population. Platelets also play important roles in the accelerated atheroscelrosis seen in diabetes as well as in the development of diabetic microangiopathy.

The pathologies of diabetic macroangiopathy are similar to those seen with atherosclerosis in nondiabetic subjects. The differences of coronary disease in diabetics as compared with nondiabetic patients are: (a) the frequency is increased; (b) the usual protective effect of female sex on coronary disease is eliminated by diabetes; and (c) the coronary disease develops at a younger age in diabetic patients than in nondiabetic patients, especially if renal disease supervenes (Kannel and McGee, 1979; Gordon et al., 1977). The data regarding peripheral and cerebral disease in diabetes are more scarce, but similarly there is a two to five fold increase in the risk of diabetic complications (Kuller, 1985; Abbott, et al., 1987).

Structurally the atherosclerosis in diabetes is not different from that seen in nondiabetic subjects, thus the pathogenesis could be similar. Atherosclerosis is a

complicated process and abnormalities of all the components of the process could lead to accelerated atherosclerosis and enhanced thrombosis in diabetes. The platelet abnormality hypothesis is based on the general finding that diabetes is associated with a hypercoagulable state (Landgraf-Leurs, et al., 1987; Bagna and Sixma, 1986) and there is considerable in vitro and in vivo evidence indicating platelet hyperactivity and endothelial dysfunction (Lorenzi and Cagliero, 1991) in both types of diabetes and in experimental diabetes. In addition, platelet hyperactivity has clinical significance. The results of the PARD (Platelet Aggregation as a Risk factor for Diabetics) study have shown that the incidence of macrovascular endpoints in male diabetic patients was strongly related to the degree of spontaneous platelet aggregation at study entry (Breddin et al., 1986).

In diabetic patients, hyperactive platelets, injured endothelial interface, increased availability of thrombotic precursors, reduced coagulation inhibitors and diminished fibrinolysis can lead to the increased propensity to vascular complications. There is abundant evidence of altered coagulation, endothelial dysfunction, platelet hyperfunction and enhanced thrombosis in vivo in diabetes mellitus.

Altered Coagulation in Diabetes

Increased Fibrinogen Concentration in Diabetes: Fibrinogen concentrations have been found to be increased in diabetes, particularly in the presence of clinical vascular

disease. Metabolic control of the diabetic subjects reduced the elevated fibrinogen concentration (Jones and Peterson, 1981).

Decreased Inhibitors of Coagulation in Diabetes: Several physiologic inhibitors of coagulation have been found to be reduced in the diabetic state. Antithrombin (AT-III) is a factor which inactivates thrombin and other coagulation factors such as factors Xa, XIa and XIIa. It has been reported that the activity of AT-III and the presence of thrombin-AT-III complexes is reduced in the plasma from both type I and II diabetic patients (Villanueva and Allen, 1988; Ceriello et al., 1990). The decreased activity of AT-III in diabetic patients was restored with more effective insulin therapy (Ceriello et al., 1990). It has also been described that in diabetic patients, plasma protein C, protein S and thrombomodulin concentrations are decreased (Conrad and Samama, 1986).

Decreased Fibrinolysis in Diabetes: It has been reported that tPA release is decreased in diabetes (Almer and Nilsson, 1975). Impaired plasmin formation has been shown in poorly controlled diabetic patients, and this condition improved after metabolic control. In vitro glycation of plasminogen showed a similar impairment of plasmin formation, although the impairment was less pronounced than when using plasminogen glycated in vivo in poorly controlled diabetic subjects (Geiger and Binder, 1988).

Endothelial Dysfunction in Diabetes

Thrombogenicity of endothelium: In vitro, it has been shown that the diabetic endothelium had a reduced capacity to secrete PGI_2, whereas thromboxane A_2 synthesis

was increased. In vivo, the production of PGI_2 and t-PA has been reported to be decreased in both type I and type II diabetic patients as well as in experimental diabetes. This diminished production of PGI_2 could interact with "sensitized" platelets to support platelet deposition on the endothelium, promoting thrombosis in the diabetic state. Insulin treatment can restore PGI_2 levels to normal as shown by some studies (Colwell et al., 1983).

vWF is an adhesion molecule produced by the endothelium. Plasma level of Von Willebrand factor (VIII R:WF) has been found to be increased in type I and type II patients and may occur in the absence of clinically apparent vascular disease, in children or in diabetic ketoacidosis in newly diagnosed patients (Colwell et al, 1981). vWF levels may also be raised as soon as 2 to 4 weeks after streptozotocin-induced diabetes in rats. These results suggest that endothelial damage is a very early event in uncontrolled diabetes. When normal cultured endothelial cells are incubated in media with a high glucose concentration, their vWF content is increased. This finding suggests that hyperglycemia has a direct effect on vWF production by endothelial cells. The elevated plasma vWF concentrations in diabetes will enhance platelet adhesion under conditions of high shear, such as in the smaller arteries and microcirculation, and thereby contribute to microvascular disease.

Endothelial function related to atherogenesis: In vitro, it has been found that endothelium cultured in a high glucose medium had increased LDL uptake, metabolism, and increased endothelial replication rate (Lorenzi and Cagliero, 1991). In vivo, diabetes

is associated with a reduced capacity of vascular relaxation, this is due to alterations in sensitivity to catecholamine and an impaired EDRF and increased production of endothelin (Hattori et al., 1991).

Endothelial injury: In humans, denuding endothelial injury may occur during certain surgical procedures, but there is recent evidence that such injury is unlikely to occur spontaneously in vivo. Injury or stimulation of the endothelium that does not result in frank endothelial loss (i.e. nondenuding injury) has been implicated in atherogenesis and has been demonstrated in the initial stages of diet-induced experimental atherosclerosis (Ross, 1986). Nondenuding endothelial injury has been considered to have occurred in vivo whenever increased synthesis of endothelial DNA is seen in the absence of morphologic evidence of endothelial cell loss.

There is evidence for endothelial injury in diabetic animals. During the first 6 months of alloxan-induced diabetes in rabbits, there is an increase in endothelial cell thymidine index, increased accumulation of white blood cells and platelets, and increased deposition of fibrin-like material on the endothelial surface, consistent with a progressive nondenuding injury (Hadcock et al., 1991). Endothelial cell denuding has not been observed at any stage of diabetes. In diabetes, mechanisms for injury to the endothelium include effects of insulin, dyslipoproteinemia, nonenzymatic glycation of proteins such as LDL and collagen, and the formation of advanced glycosylation end products.

Platelet Hyperactivity in Diabetes

Platelet adhesion: Platelet adhesion to the vessel wall is the initial event in thrombogenesis. It has been shown that platelet adhesion, measured as retention on glass bead columns, was increased in diabetic patients (Shaw et al, 1967; Mayne et al., 1970; Heath et al., 1971) and was more evident in those with vascular complications (Hellem, A.J., 1971). Hyperglycemia has been suggested to have a direct effect on platelet adhesion. Oral or intravenous glucose administration in vivo and in vitro glucose addition have been found to increase platelet adhesion, as measured by platelet stickiness to a glass surface (Bridge et al., 1965).

Platelet aggregation: There is a lot of evidence demonstrating that platelet aggregation in response to aggregating agents (ADP, collagen, arachidonic acid, thrombin, PAF) is increased in platelet-rich plasma, washed platelets, and whole blood from type I and type II diabetic patients (Kwaan et al., 1972; O'Malley et al. 1975; Creter et al.,1978; Khosla et al., 1979). Increased platelet aggregation can be found in diabetic children (Colwell et al., 1990), "latent" diabetic patients and in diabetic patients without detectable vascular disease (Sagel et al.,1975). Platelet hyperactivity has also been found in diabetic animals (mainly rats) and it preceded vessel wall change which was detected by an increase of plasma vWF concentration (Colwell et al., 1990). These results indicate that in diabetes platelet hyperactivity occurs before the detectable vascular changes and therefore contributes to the pathogenesis of diabetic vascular complications.

The etiology of enhanced platelet aggregation may be multifactorial. Jones et al., (1986) performed multiple regression analysis of epidemiological factors and found that only blood glucose was significantly correlated with platelet aggregation. Kobbah et al., (1989) also showed that platelet aggregation was increased even in newly diagnosed type I diabetes mellitus but after insulin treatment, the increased platelet aggregation returned to normal. These studies indicate that the metabolic abnormality of diabetes mellitus is one factor causing the enhanced platelet aggregation in diabetes. Other factors include insulin and an unknown plasma factor. Trovati et al., (1988) showed that addition of insulin in vitro and in vivo reduced platelet sensitivity to aggregating agents. Other investigators have found that in diabetic patients, there are some plasma factors known as "platelet aggregation enhancing factors" (Neri, et al., 1980) which potentiate platelet aggregation. The precise nature of these factors is not known. On the other hand, a washed platelet suspension from diabetic subjects also showed increased aggregation indicating that platelets per se have the property of hyperaggregability. It has been suggested (Gensini et al., 1979) that plasma factors may modify the hyperaggregability of platelets from diabetic patients and may be related to the discrepancies from different studies and between platelet-rich plasma and washed platelets. Platelet hyperaggregability in early diabetes mellitus may be mainly due to primary platelet hypersensitivity, while in the advanced stage of the disease which comprises vascular complications, the platelet hyperaggregability may be due to plasma factors.

Platelet release: It is unclear whether platelets from diabetic subjects release more of their granule contents in response to agonists (Winocour and Richardson, 1993).

On one hand, it was reported that release of amine contents from platelets from diabetic individuals with or without retinopathy was similar to that in control subjects. On the other hand, it was shown that washed platelets from diabetic rats release more serotonin from their amine storage granules after thrombin or collagen.

In vivo evidence: Because assessment of platelet function in vitro may not accurately reflect the behavior of platelets in vivo, platelet function in vivo has also been estimated (Bern, 1978; Colwell et al., 1981; Colwell et al., 1983).

One way to evaluate platelet function in vivo is to measure the plasma or urine concentrations of the materials released from activated platelets. Beta-thromboglobulin (bTG) and platelet factor 4 (PF_4) are platelet-specific proteins which are stored in platelet alpha granules and released during platelet activation. Therefore plasma levels of these proteins can reflect in vivo platelet activation. Most studies have reported that in diabetic patients, the plasma concentrations of bTG and PF_4 are increased (Ishii, 1992). It has also been suggested that metabolic disturbances may be responsible for the elevation of bTG and PF_4, which could be reversed by metabolic control. Diabetic vascular complications may provoke increased platelet activation to release the platelet-specific proteins.

Another approach to assess platelet behaviour in vivo is to determine how long platelets survive in the circulation. Platelet survival reflects the balance between the rates of production of platelets from megakaryocytes and of clearance of platelets from the circulation either by the reticuloendothelial system or through platelet-vessel wall interactions. It has been observed that platelet survival is shortened in diabetic subjects

compared with nondiabetic controls (Colwell et al., 1988). It was also found that the peripheral platelet volume distribution in diabetics is broadened and particularly shifted to larger platelets as compared with that in healthy controls, whereas the platelet count remains the same (Tschoepe et al., 1991). One interpretation for this finding is that platelet consumption is enhanced at injured endothelial interfaces, which promotes the new entry of young and large platelets.

In addition another index reflecting the degree of in vivo platelet aggregation (circulating platelet aggregates) has been shown to be increased in type I and type II diabetic patients, confirming the results of in vitro studies (Davis et al., 1982; Dallinger et al., 1987).

Enhanced thrombosis in vivo

In diabetic retinopathy, microaneurysms and small areas of capillary occlusion as identified by fluorescein angiopathy are the earliest and most characteristic clinical findings in diabetic retinopathy. Microaneurysm formation appears to be related to capillary occlusion (Kohner and Sleightholm, 1986). Since it has been shown that in the arterial circulation acute thrombotic events are predominantly triggered by platelets (Fitzgerald et al., 1986; Martin et al., 1991; Mizuno et al., 1992), platelet aggregates may produce capillary microembolization, promoting the early evolution of microangiopathic organ lesions such as retinal capillary closure.

Indeed, platelet aggregates and thrombi have been observed to exist in the small vessels of the retina and endoneural vessels of diabetic patients and animals (Doddle et al., 1974; Ishibashi et al., 1981). The thrombi appear to consist mainly of platelets and fibrin. Platelet microthrombi have also been found to exist in the microvasculture of sural nerves from diabetic patients with neuropathy (Williams et al., 1980) and in the cerebral circulation of patients with diabetic ketoacidosis (Timperly et al., 1974) . These studies support the hypothesis that platelet dysfunction may contribute to microocclusive vascular disease in diabetes.

Capillary damage in the glomerulus and basement membrane thickening are the morphological features (Mogensen et al., 1979) of diabetic nephropathy. The increased width results mainly from hyperglycemia-induced crosslinking of matrix protein (Williamson and Kilo, 1984) such as collagen and carbohydrates. Platelets are also a potential source of mediators possibly affecting capillary permeability, mesangial function and glomerular haemodynamics. Moreover, platelets contain growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). It has been suggested that similar to their roles in atherosclerosis, platelets might contribute to glomerular lesions by inducing mesangial cell proliferation with excessive generation of mesangial matrix. Recently, Nakamura et al., (1993) quantified the mRNA expression for various growth factors in control and diabetic rats and found an increase in tumor necrosis factor-a (TNF-a), PDGF, TGF-beta, and bFGF in diabetic rats.

There is also evidence that thrombosis in large vessels in response to injury occurs more readily in diabetics than in non-diabetic individuals. It was observed that in diabetic rabbits, ADP-induced platelet thrombus formation was increased in response to electrical injury to pial arteries (Honour and Hockaday, 1976). The presence of platelets and microthrombi was reported at sites of endothelial cell loss in aortas of diabetic rats (Arbogast et al., 1984). Increased platelet accumulation also occurred in aortas repeatedly damaged for 4 days with indwelling catheters in genetically determined diabetic rats, although macroscopic thrombi did not form in either control or diabetic rats (Winocour et al., 1987).

II.2 Mechanisms of Platelet Hyperactivity (Statement of Problem)

Receptor

Since most agonists activate platelets via interaction with their specific receptors on the platelet membrane, it is possible that platelet hyperfunction in diabetes mellitus could be a result of altered receptor activity. It has been reported (Tschope et al.,1990) that the number of GPIb and GPIIb/IIa molecules per platelet (the receptors for vWF and fibrinogen) is increased in both type I and type II diabetic patients. It has also been shown that in response to agonists, fibrinogen binding to platelets is increased in diabetic subjects (DiMinno et al., 1985). In addition, plasma concentrations of fibrinogen are elevated in patients with diabetes (Kannel et al., 1987). However, the results from other receptors on platelet plasma membranes are conflicting with platelet hyperfunction in diabetes. For example, the number of binding sites for TXA_2 receptor in platelets from type I diabetic

patients was decreased or unchanged (Ishii et al., 1992). The alteration of thromboxane receptors may be due to a down-regulation induced by the increased production of TXA_2. The number of platelet alpha-adrenergic receptors was decreased in type I diabetic patients with autonomic neuropathy (Ishii et al., 1992). These results suggest that the increased platelet sensitivity to TXA_2 and epinephrine may be derived from changes in post-receptor process.

Platelet Membrane Lipid fluidity

Platelet hyperactivity in diabetes could result from changes in membrane fluidity which modulates cell function. It has been reported that the membrane fluidity is decreased in the hypersensitive platelets from poorly controlled type I and II diabetic patients (Winocour et al., 1990). This reduced membrane fluidity correlated with an increased extent of glycation of membrane proteins, whereas the cholesterol/phospholipid molar ratio was similar in platelet membranes from control and diabetic subjects. When isolated platelet membranes from nondiabetic subjects were incubated in a medium of high glucose, which was close to the glucose level in poorly-controlled diabetic patients, the membrane lipid fluidity was decreased and there was an increase in the extent of glycation of membrane proteins (Winocour et al., 1992). These results suggest that increased glycation of membrane proteins plays a role in the hypersensitivity of platelets from diabetic patients.

Arachidonate pathway

Thromboxane A2 (TXA_2) is a signal amplifier for most platelet activators. It is generally reported that TXA_2 production is increased in diabetic animal models and type I and type II diabetes mellitus (Ishii, et al., 1992). There is a significant correlation between TXA_2 production and fasting plasma glucose or HbA_{1c} values (Abbate et al., 1988). Furthermore, TXA_2 production can be restored by subcutaneous insulin infusion. These findings clearly suggest that enhanced TXA_2 production in diabetes mellitus may be attributable to a metabolic disorder.

Plasma or urinary concentrations of TXB_2, which is a stable metabolite of TXA_2, have also been found to be increased in diabetic subjects, indicating an enhanced activation of platelet arachidonate pathway in diabetic subjects in vivo, since the concentration of TXB_2 could not be affected by artifactual TXA_2 production.

Nonarachidonate Pathways

Based on the hypothesis that platelets are involved in the enhanced vascular disease seen in diabetic patients, approaches to modify the function of these altered platelets should be clinically important. If enhanced activity of the arachidonate pathway is responsible for the platelet hypersensitivity in diabetes, then the use of aspirin as a platelet inhibitor should have beneficial outcomes. During the last 10 years, several clinical trials of antiplatelet therapy (mainly using aspirin) have been carried out, but the clinical outcome has not been satisfactory (Colwell, 1992; Patrono and Davi, 1993).

One explanation for the limited beneficial outcomes of these clinical trials may be that mechanisms other than the arachidonate pathway are also altered in platelets from diabetic patients. In vitro studies also support this hypothesis because it has been shown that the platelet hyperactivity to thrombin in diabetes is independent of the activation of the arachidonate pathway and the effects of released ADP (Colwell et al., 1990). When platelets were pretreated with aspirin, which inhibits cyclooxygenase and creatine phosphate-creatine phospholkinase (CP/CPK) which removes ADP, the platelet hyperactivity to thrombin still persisted in diabetes (Winocour et al., 1986a; 1986b). It was also found that the amount of thrombin that bound to its receptor on platelets from diabetic rats was similar to that seen in control rats (Winocour et al., 1991).

All this evidence suggests that, in platelets from diabetics nonarachidonate post-receptor mechanisms in platelet activation are also responsible for the platelet hypersensitivity to thrombin. Among the post-receptor pathways one possibility is that signal transduction mechanisms are altered in diabetes. As mentioned in the general review of platelets, cytosolic calcium plays a crucial role in intra-platelet signal transduction mechanisms and is a critical step in platelet reactions, including shape change, secretion and aggregation. Also it has also been shown that cytosolic calcium is disturbed in a variety of tissues in diabetes (Carafoli, 1987). Therefore, it is worthwhile to investigate whether cytosolic calcium handling by platelets is affected in diabetes mellitus. In the literature, there are many reports about the various abnormalities of platelet function in diabetes, but there are only a few studies that report abnormalities in calcium homeostasis in platelets from diabetic subjects. The results from these studies are not

consistent. In addition, no study has identified the mechanisms underlying the abnormality of calcium homeostasis in platelets from diabetics.

Therefore, the objective of the first part of this project was to determine whether platelet calcium homeostasis is deranged and if so, to investigate the mechanism(s) for the calcium disturbances.

PART III. PLATELET CALCIUM HOMEOSTASIS

Many platelet agonists such as thrombin, ADP, TXA_2, vasopressin and PAF can induce an increase in cytosolic Ca^{2+} concentration. This $[Ca^{2+}]_i$ rise is generated from both Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular medium. The increased cytosolic Ca^{2+} is then removed by Ca^{2+} efflux into the extracellular medium or reuptake into the internal stores.

Ca^{2+} homeostasis is a complex process modulated by many regulatory mechanisms controlling Ca^{2+} influx, efflux, sequestration, and release from intracellular stores. The normal resting cytosolic calcium levels can be maintained in the presence of a 10^4 fold concentration gradient across the membrane and the cells can produce Ca^{2+} signals with temporal and spatial characteristics in response to various stimuli.

III.1 CALCIUM RELEASE FROM THE INTRACELLULAR STORE

It has been shown that platelets still function in the absence of external Ca^{2+}, suggesting that there is an intracellular Ca^{2+} store in platelets (Rink, et al., 1982). The

observation that Ca^{2+} ionophores can activate platelets in the absence of external Ca^{2+} provides direct evidence of a pool of Ca^{2+} sequestered in intracellular organelles. In quin-2-loaded platelets suspended in Ca^{2+}-free medium, agonists can evoke $[Ca^{2+}]_i$ transients, confirming the existence of receptor-mediated internal Ca^{2+} discharge.

In platelets, the dense tubular system (DTS) is the main storage site of intracellular Ca^{2+}, since it has been shown to accumulate Ca^{2+} in an ATP-dependent manner within the range of $[Ca^{2+}]_i$ thought to exist in the platelet cytosol (Menashi et al., 1984; O'Rourke et al., 1985). Addition of mitochondria uncouplers to human platelets does not evoke store-discharge or prevent IP_3-generating agonists from releasing the internal Ca^{2+} pool, suggesting that the mitochondria are not a significant source of releasable Ca^{2+} (Brass, 1984).

The total Ca^{2+} content in platelet internal Ca^{2+} stores has been estimated to be about 10-20mM, however the free Ca^{2+} concentration in the store may be in the μM range, since most of the Ca^{2+} in the store is bound to a calsequestrin-like molecule (Rink et al., 1982; Volpe et al., 1988). Agonist-evoked intracellular Ca^{2+} store discharge can increase $[Ca^{2+}]_i$ to at least 1 μM (Pollock et al., 1986) which is within the range of $[Ca^{2+}]_i$ expected to influence target proteins.

IP_3 is the link between agonist-receptor binding and Ca^{2+} release from internal stores in platelets. In platelets, IP_3 is produced from PIP_2 hydrolysis after PLC activation that results from the interaction of some agonists (such as thrombin, TXA_2, PAF, and vasopressin) with their respective receptors. It has been shown that IP_3 (1-10μM) can

cause Ca^{2+} release from platelet microsomes and permeabilized platelets (Rengasamy and Feinberg, 1988). Similar to many other cells, the mechanism of IP_3-induced intracellular calcium release is via the IP_3 receptor in the intracellular store that is closely associated with a Ca^{2+} channel (Berridge and Irvine, 1984). The IP_3 binding site (or receptor) has been characterized in platelets and in many other tissues with a K_d ranging from 0.1-80 nM (see review of Ferris and Snyder, 1992). The receptor for IP_3 has been purified (Supattapone et al., 1988) and cloned (Furuichi et al., 1989) from a number of tissues, but not from platelets. Although it may be be assumed that the receptor in platelets has similar properties to those described in other tissues, the fine regulation of IP_3 receptor (IP_3R) may be different. There are at least 3 and probably 4 genes that code for the IP_3R (Ross et al., 1992). The isotype of IP_3R expressed in platelets is not known. By using peptides derived from the cloned receptor and monoclonal antibodies it has been shown that sequences near the N terminal region bind IP_3 and that sequences near the C terminal end represent the channel portion of the molecule (Nakade et al., 1991). After IP_3 interacts with its receptor in the intracellular store, the Ca^{2+} channel opens and Ca^{2+} is released into the cytoplasm .

In addition, in platelets there may be some IP_3-independent mechanisms for Ca^{2+} release from internal stores following agonist stimulation. In the absence of external Ca^{2+}, ADP can evoke internal Ca^{2+} discharge within 200 msec, but ADP evokes minimal (Daniel et al., 1986) or no (Fisher et al., 1985) inositol phosphate hydrolysis and IP_3 formation (Siess, 1989). This finding indicates that there are alternative pathways for internal Ca^{2+} discharge, however the mechanism for this pathway has not been identified.

In some cell types (e.g. muscle) the major Ca^{2+} release channel is the ryanodine receptor. The activity of this receptor can be modulated by the plant alkaloid, ryanodine (Fleischer and Inui, 1989) thus it has been called the ryanodine receptor. Ca^{2+} can activate these receptors through a process called calcium-induced calcium release (CICR). Recently it has been suggested that cyclic ADP-ribose (cADPR) may be acting in sea-urchin eggs through a CICR mechanism mediated by ryanodine receptors (Galione, 1992), suggesting that cADPR may be an endogenous releaser of stored Ca^{2+}. cADPR is a low molecular weight, endogenous metabolite of the pyridine nucleotide (NAD^+). It is formed by the action of ADP-ribosyl cyclases. cADPR can be found in many mammalian and invertebrate tissues (Dargie et al., 1990). cADPR has been shown to be as potent as IP_3 at releasing Ca^{2+} in sea-urchin eggs (Clapper et al., 1987). Whether or not the ryanodine receptor exists in platelets is not known.

III.2 Calcium Influx

When an agonist activates platelets, in addition to the receptor-mediated discharge of the intracellular stores, there is Ca^{2+} entry. Agonist-stimulated Ca^{2+} entry is supported by the following evidence: (a) increased uptake of ^{45}Ca after stimulation; (b) a much larger $[Ca^{2+}]_i$ response in quin-2 loaded platelets in the presence than in the absence of external Ca^{2+}. (c) stimulated entry of Mn^{2+} detected by the ability of this ion to quench quin-2 or fura-2 fluorescence; and (d) stopped flow fluorescence measurements showing an earlier agonist-evoked $[Ca^{2+}]_i$ signal in the presence rather than in the absence of external Ca^{2+} (Sage and Rink, 1986b; 1989; Sage et al., 1990).

Calcium entry can provide a faster and more prolonged $[Ca^{2+}]_i$ rise signal than that provided by internal release only. The following are some possible mechanisms for platelet Ca^{2+} entry.

Receptor-mediated Ca^{2+} entry

In platelets, agonist-stimulated Ca^{2+} entry is due to receptor-mediated events. There are several mechanisms for receptor-mediated Ca^{2+} entry: (a) receptor-operated channels (ROC's) in which the agonist-receptor binding is directly coupled to channel opening; (b) second messenger-operated channels (SMOC) which are opened by a diffusible messenger itself produced as a result of agonist receptor binding; (c) store-regulated Ca^{2+} entry which is the Ca^{2+} entry regulated by the state of filling of the intracellular store (Putney, 1990); and (d) Ca^{2+} could enter the cell in exchange for, or coupled with, other ions following agonist-evoked activation of a carrier in the plasma membrane. After agonist stimulation, any of these Ca^{2+} entry mechanisms is possible and several may operate simultaneously or sequentially in platelets.

Receptor-Operated Channel (ROC): The first evidence that the ADP receptor was tightly coupled to the opening of a membrane channel came from the stopped-flow technique, which allows detection of fluorescence changes on a 10-ms time scale (Sage and Rink, 1987). By using this technique it was found that ADP evokes a very rapid Ca^{2+} influx in quin-2 or fura-2 loaded platelets. This ADP-evoked Ca^{2+} influx occured without

measurable delay (less than the mixing time of 30 ms). The rapidity of ADP-evoked influx suggests a close coupling between the receptor occupation and the Ca^{2+} channel.

Direct evidence of ADP-evoked ROC derives from patch clamp studies. In whole cell recordings, Maruyama (1987) recorded voltage-gated K^+ channels in unstimulated platelets but was unable to maintain stable recordings only after the addition of agonists to the bath. In recordings from cell attached membrane patches, Mahaut-Smith, Sage and Rink (1990) observed activation of single-channel currents when ADP was included in the pipette but not when added to the bath. This result agrees with the conclusion from the stopped-flow fluorescence experiments that the ADP-evoked channel was ligand-gated and did not require the involvement of a diffusible second messenger. The ADP-evoked channel is a nonselective cation-permeable channel, conducting sizable amounts of Na^+ and Ca^{2+} under physiological conditions. It is similar in many respects to the ATP-activated channel in smooth muscle cells.

Second Messenger Operated Channel (SMOC) In platelets, only ADP has been identified to activate ROC. Other agonists such as thrombin, platelet-activating factor, vasopressin and TXA_2 analogue U-46619, evoke Ca^{2+} influx with an irreducible delay of at least 200 ms in the stopped-flow experiments (Sage and Rink, 1987). These data suggest that there are several biochemical steps between receptor occupancy and generation of Ca^{2+} entry by these agonists.

One explanation is that agonists activate the receptor, generating a diffusible second messenger that opens a Ca^{2+}-permeable channel in the plasma membrane. Ca^{2+} and inositol phosphates are the two possible second messenger candidates in channel gating.

Calcium-sensitive Ca^{2+} channels have been proposed to mediate Ca^{2+} influx in human neutrophils, but such a mechanism may not operate in platelets (Sage and Rink, 1987). Stopped-flow studies have indicated that the $[Ca^{2+}]_i$ increment is faster in the presence than in the absence of extracellular Ca^{2+}, suggesting that Ca^{2+} influx is not the result of $[Ca^{2+}]_i$ elevation (Sage and Rink, 1987; Sage et al., 1989; 1990b). Moreover, Ca^{2+} entry was preserved in platelets heavily loaded with quin-2 to buffer the rise in $[Ca^{2+}]_i$ caused by internal release. Kimura et al., (1993) also showed that Ca^{2+} influx was activated at low levels of $[Ca^{2+}]_i$.

Until recently there has been only one report concerning a direct role for IP_3 in generating Ca^{2+} influx in platelets. In this study, it was found that IP_3 released Ca^{2+} from membrane vesicles enriched in plasma membrane (Rengasamy and Feinberg, 1988). Another possibility is that IP_4 may act in concert with IP_3 to generate Ca^{2+} entry. However, there is no direct evidence for a role of IP_4 in platelets.

Although a lot of evidence indicates that thrombin can evoke Ca^{2+} influx from the extracellular medium, surprisingly the thrombin-induced Ca^{2+} influx cannot be detected electrophysiologically. In patch clamp studies, applying thrombin to platelets for 3 sec produced no inward current, but under the same conditions ADP produced typical inward currents. To determine whether there is Ca^{2+} entry during the time course of

thrombin application, stopped-flow experiments were performed in the same conditions. It was found that thrombin can evoke a $[Ca^{2+}]_i$ rise after a delay of 1.1s and there is Mn^{2+} entry after a delay of 1.4s (Sage and Rink, 1987).

There are a few possibilities to explain why the early thrombin-evoked Ca^{2+} entry cannot be detected electrically. (1) Generally, most receptor-operated and second messenger-operated channels characterized to date are relatively nonselective cation channels that conduct significant amounts of Na^+ and Ca^{2+}. The thrombin-evoked channel may be an exception. The thrombin-evoked channel could be a highly selective divalent cation channel and therefore is beyond the resolution of currently available patch-clamp recordings. (2) Thrombin may activate an electroneutral mechanism that enables Ca^{2+} to enter the cell in exchange for K^+ or H^+ or by cotransport with Cl^- (Sage et al., 1992a). (3) The thrombin-evoked current is already activated under patch clamp conditions. (4) Thrombin failed to cleave its receptors and activate an elevation in $[Ca^{2+}]_i$. This issue has so far not been resolved.

Store-regulated calcium entry was also referred to as the "capacitative" model and was first proposed by Putney (1986). This model suggests that that Ca^{2+} entry is somehow regulated by the filling state of intracellular Ca^{2+} stores. Emptying of IP_3-sensitive stores would send a signal to the plasma membrane to increase its Ca^{2+} permeability and thereby replenish the store (Putney, 1990).

This entry pathway has been directly demonstrated by showing that depletion of intracellular stores, by itself, leads to the activation of a Ca^{2+} current. To distinguish it

from other Ca^{2+} entry channels, it has been named the Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}), meaning the current flowing through these capacitive Ca^{2+} entry channels (Hoth and Penner, 1992). It has been suggested that I_{CRAC} passes through a channel rather than a carrier (Zwefsch and Lewis, 1993). However, the nature of the permeation mechanism for I_{CRAC} is still unknown. For convenience, it has been referred to as a CRAC channel, reminding the fact that this remains to be proven. Until now, the CRAC channel has not been biochemically identified. The cation selectivity of the CRAC channel is: $Ca^{2+}>Ba^{2+}>Sr^{2+}>>>Na^{+}$.

Several models have been proposed to explain the coupling between the Ca^{2+} content of the intracellular stores and the regulation of a plasma-membrane Ca^{2+} channel. Putney (1986) initially proposed that there is a direct coupling between Ca^{2+} stores and the plasma membrane, possibly via a "gap junction-like" structure. This seems unlikely because there is no strong morphological evidence supporting this hypothesis.

Another model is that an unknown cytosolic factor is released from intracellular stores after the decrease of luminal Ca^{2+} concentration (Putney, 1990) and then diffuses to the plasma membrane to open I_{CRAC}. There are a few candidates for this factor. One is CIF (Ca^{2+}-influx factor). CIF is a low-molecular-mass phosphorylated compound that is stored in the ER awaiting the onset of store depletion for its release. It was partially purified from Jurkat T cells where I_{CRAC} was first described (Randriamampita and Tsien ,1993). The identity of CIF has not been determined.

In addition to CIF, other factors have been reported to modulate I_{CRAC} or Ca^{2+} influx induced by store depletion. These include G proteins (Fasolato et al., 1993), IP_3 and IP_4 (Berridge, 1993), cytochrome P-450 (Alvarez et al., 1991), tyrosine kinase and protein kinase C (Sargeant et al., 1993).

Recently a new model has been proposed (Berridge, 1995) to explain how the internal stores can regulate the CRAC channels in the plasma membrane. In this model, it is proposed that the information may be transferred through a protein-protein interaction such as phosphorylation or conformational coupling.

The store-regulated Ca^{2+} entry mechanism has been demonstrated to be present and have similar properties in many cells types (Putney, 1990). In platelets, this Ca^{2+} entry mechanism may contribute, to a greater or lesser extent, to the Ca^{2+} influx generated in response to all platelet agonists which, in turn, releases Ca^{2+} from intracellular stores. For example, ADP evokes Ca^{2+} entry via two pathways: a ROC and a store-regulated pathway (Sage et al., 1990b; Mahaut-Smith et al., 1990, 1992). As far as thrombin evoked Ca^{2+} influx is concerned, there are 2 explanations. One is that the store-regulated pathway may account for all the thrombin-evoked Ca^{2+} entry. The second is that there are 2 portions of the Ca^{2+} influx pathway for thrombin. One pathway precedes store release and may be second messenger mediated, but electrophysiologically undetectable. The second pathway might be the store regulated pathway (Sage et al., 1992). By using a fluorimetry method Hashimoto et al.,(1992) showed that thrombin activates 2 different Ca^{2+} entry pathways in human platelets: an early and a late thrombin-activated Ca^{2+} influx

(TE and TL, respectively). It is suggested that a TL Ca^{2+} channel is responsible for the plateau phase of the thrombin-evoked $[Ca^{2+}]_i$ rise, whereas a TE Ca^{2+} channel mediates the early component of thrombin-evoked Ca^{2+} entry.

Lack of Voltage-gated calcium channels

Voltage-operated Ca^{2+} channels do not appear to mediate agonist-evoked Ca^{2+} entry in platelets. A lot of evidence supports this point. Similar to other cells, platelets have concentration gradients of Na^+ and K^+ across the plasma membrane, which is maintained by a ouabain-sensitive Na^+-K^+- ATPase. The resting membrane potential of platelets is estimated to be around -70 mV by fluorescent carbocyanine dyes (MacIntyre and Rink, 1982; Pipili, 1985) and radiolabelled membrane permeant ions (Wencel-Drake and Feinberg, 1985). Thrombin, ADP and platelet-activating factor (PAF) have been shown to cause depolarization of the platelet membrane (Horne and Simons, 1978; Greenberg-Sepersky and Simons, 1984; Pipili, 1985), but the changes do not correlate well with platelet activation. The depolarization is only 4-10 mV (Wencel-Drake and Feinberg, 1985; Pipili, 1985) which is less than that required to promote voltage-gated Ca^{2+} entry.

High K^+ solutions depolarize but do not activate platelets (MacIntyre and Rink, 1982; Doyle and Ruegg, 1985; Hallam and Rink, 1985a) and it has been shown qualitatively that substitution of external Na^+ for non-permeant cations does not affect the response to thrombin (Connolly and Limbird, 1983b; Pipili, 1985). Depolarization of the platelet membrane in high-K^+ solutions is without effect on basal $[Ca^{2+}]_i$ and reduces,

rather than increases, agonist-evoked rises in $[Ca^{2+}]_i$ (Sage and Rink, 1986c). Agonist evoked Ca^{2+} signals are also reduced when the membrane is depolarized with gramicidin, suggesting that the effect is due to a reduced driving force for Ca^{2+} entry (Mahaut-Smith et al, 1990).

Organic Ca^{2+} channel antagonists have relatively little effect on agonist-evoked $[Ca^{2+}]_i$ rise in platelets, at least at concentrations that block Ca^{2+} entry in excitable cells. Higher concentrations are reported to have inhibitory effects (Avodin et al., 1988; Hallam, 1985), but this may result from membrane depolarization due to an action of these agents on K^+ channels (Maruyuma, 1987). There are no detectable binding sites for verapamil and nitrendipine on human platelets (Motulsky et al, 1983), while such sites are readily found on cells with well-documented voltage-dependent Ca^{2+} channels such as smooth or cardiac muscle. Interestingly, the platelet plasma membrane has voltage-gated K^+ channels (Maruyama, 1987).

III.3 CALCIUM SEQUESTRATION

The reuptake of cytosolic Ca^{2+} into intracellular stores by the Ca^{2+} -ATPase in the sarco/endoplasmic reticulum plays an important roles in the Ca^{2+} homeostasis of many cells. It has been found that at least three genes encode the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (MacLennan et al., 1985) . SERCA has a molecular weight of 100-115 KDa. $SERCA_1$ Ca^{2+}-ATPase isoform is expressed in fast skeletal muscle. $SERCA_2$ contains $SERCA_{2a}$ and $SERCA_{2b}$ which are produced by alternative splicing. $SERCA_{2a}$ is expressed in cardiac and slow skeletal muscle, while $SERCA_{2b}$ is expressed in

smooth muscle and several non-muscle cells. $SERCA_3$ is present in several muscle and non-muscle tissues.

In platelets the Ca^{2+}-ATPase is located in the dense tubular system (DTS). Dense tubules have active Ca^{2+} pumping systems similar to sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and 2 separate Ca^{2+}-ATPase have been identified in platelets (Papp et al., 1991). Recently it has been shown that platelets express both $SERCA_{2b}$ (Enouf et al., 1992) and $SERCA_3$ Ca^{2+}-ATPase isoforms. It is not clear why different Ca^{2+}-ATPase isoforms coexist in platelets. It is possible that in platelets there are 2 functionally distinct Ca^{2+} storage pools where 2 different isoforms are located.

III.4 Calcium Efflux

Generally the plasma membrane of mammalian cells has 2 different Ca^{2+} extrusion mechanisms: the plasma membrane Ca^{2+} ATPase (PMCA) and the Na^{+}-Ca^{2+} exchanger. PMCA is an ATP-driven pump which actively extrudes Ca^{2+} out of the cell and is not reversible. The Na^{+}-Ca^{2+} exchanger is a reversible carrier mechanism.

The role of the plasma membrane Ca^{2+}-ATPase in platelet Ca^{2+} homeostasis has been controversial. The plasma membrane Ca^{2+}-ATPase has been well established in many other cells, as reviewed by Carafoli (1992). It has a molecular weight of 124-136 KDa and humans have 4 PMCA genes: PMCA1, PMCA2, PMCA3 and PMCA4. PM Ca^{2+}-ATPase can be stimulated by calmodulin and inhibited by vanadate. However, until now, no definitive demonstration of a PM Ca^{2+}-ATPase in platelets has been described.

Some initial studies using membranes of varying purity, suggested that PM Ca^{2+}-ATPase exists in platelets but other studies using highly purified plasma membrane fractions obtained by different techniques failed to confirm these observations. For example, Enyedi et al., (1986) found that there may be 2 distinct Ca^{2+}-ATPase activities in platelets. However when highly purified plasma membrane fractions were used, the Ca^{2+}-ATPase activities couldn't be detected suggesting that both Ca^{2+}-ATPase activities are present on intracellular membranes (Hack et al.,1986). Recently, Papp et al., (1991) did not find a PM Ca^{2+}-ATPase protein in platelet membranes by using a specific antibody for the 135 KDa PM Ca^{2+}-ATPase. These studies suggest that other Ca^{2+} extrusion mechanisms (Na^{+}-Ca^{2+} exchanger) may be more important in platelet Ca^{2+} homeostasis..

Na^{+}-Ca^{2+} exchanger activity has been demonstrated to exist in human platelet plasma membrane vesicles (Rengasamy et al., 1987). However, the results in intact platelets are not consistent. Some studies found that changing the Na^{+} gradient and/or pretreating platelets with ouabain had some effects on cytosolic calcium (Schaeffer, 1989; Lees, 1989). However, other studies did not demonstrate such an effect (Rink, 1987; Zimlichman, 1987; Rink, 1988). These inconsistent data make the existence of a Na^{+}-Ca^{2+} exchanger and its role in platelet Ca^{2+} homeostasis controversial. Recently, it has been shown that human platelets displayed Na^{+}-Ca^{2+}, K^{+} exchange activity (Kimura et al., 1993), but its relationship to the retinal rod exchanger has yet to be established. In addition, the physiological function of the Na^{+}-Ca^{2+} exchanger in platelets is not clear. Therfore the objective of the second part of this project was to first confirm the existence of a Na^{+}-Ca^{2+} exchanger in platelets and then to determine its physiological role. We

then determined whether this Na^+-Ca^{2+} exchanger is changed in diabetes. In diabetes, if platelet calcium homeostasis is disturbed, all the regulatory pathways including Ca^{2+} influx, release, efflux, resequestration could be abnormal. The Na^+-Ca^{2+} exchanger has some characteristics that suggest that this exchanger might be a candidate leading to abnormal platelet calcium homeostasis in diabetes.

PART IV. SODIUM CALCIUM EXCHANGER

IV.1 BASIC CHARACTERISTICS

The Na^+-Ca^{2+} exchanger is an important transport system in the plasma membrane that transports Ca^{2+} bidirectionally. It can mediate Ca^{2+} efflux in exchange for extracellular Na^+ (forward mode), or promote Ca^{2+} influx in exchange for intracellular Na^+ (reverse mode). Na^+-Ca^{2+} exchange activity was first measured functionally as a Na^+-dependent Ca^{2+} flux in the mid-1960s in mammalian cardiac muscle and in squid axons (Baker et al., 1969). It has now been functionally and biochemically recognized that there are two types of Na^+-Ca^{2+} exchanger. One is the cardiac type, which has a stoichiometry of 3 Na^+:1 Ca^{2+} (Reeves and Hale, 1984; Kimura et al., 1987). The second type is the retinal rod type, in which K^+ is co-transported with Ca^{2+},with a stoichiometry of 4 Na^+:(1 Ca^{2+} + K^+) (Schnetkamp et al., 1989; Cervetto et al., 1989). In both types of exchanger and in the Ca^{2+} influx or efflux modes of exchange the Na^+-Ca^{2+} exchanger is electrogenic and voltage sensitive.

The Na^+-Ca^{2+} exchanger is a carrier mechanism. The driving force for the exchanger is the chemical gradient across the cell membrane, together with the membrane potential. The direction of net Ca^{2+} flux is governed by the electrochemical gradients of Na^+ and Ca^{2+} and the membrane potential (V_m). In addition, the direction of the transport is reversible. Under certain conditions, the exchanger may function in the Ca^{2+} influx mode.

IV.2 MOLECULAR BIOLOGY

To date, three members of the Na^+-Ca^{2+} exchanger gene family have been cloned: NCX1; NCX2; and the retinal rod outer segment Na^+-Ca^{2+}, K^+ transporter as reviewed by Philipson et al., (1996).

NCX 1 (the cardiac-type exchanger)

The Na^+-Ca^{2+} exchanger protein was identified in 1988 (Philipson, 1988) and NCX1 was first cloned from canine cardiac sarcolemma in 1990 (Nicoll et al., 1990). The NCX1 is a glycosylated multipass membrane protein which contains a cleavable leader peptide followed by 5 putative transmembrane regions at the N-terminus, 6 transmembrane regions at the C-terminus and a large intracellular loop in between (Nicoll et al., 1991). The membrane domain is responsible for ion transport, whereas the cytosolic domain plays a regulatory role and provides the point of interaction with the cytoskeleton (see figure 1).

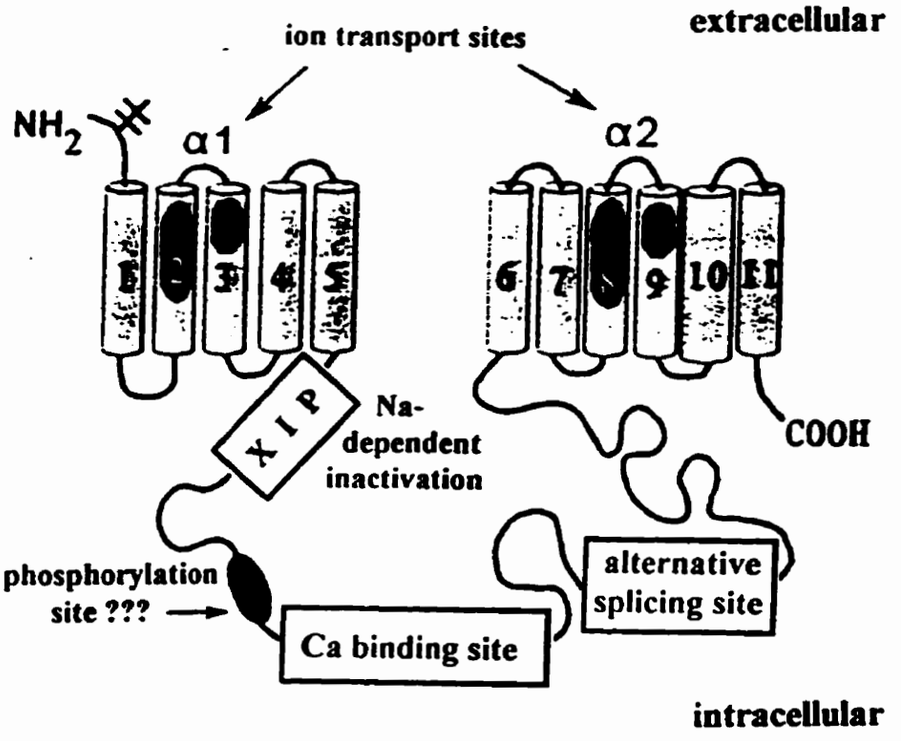


Figure 1

A model of the mammalian heart Na^+-Ca^{2+} exchanger quoted from Iwata et al.(1996). The deduced sequence predicts 11 putative transmembrane domains (TM) and a long intracellular loop after TM5. The N-terminal signal peptide might be cleaved during the posttranslational processing. Sequence comparison studies have shown that the regions of TM4 and 5 facing the extracellular surface are homologous to those of the Na/K-ATPase and TM2,3, and 8,9 to those of the Na^+-Ca^{2+}, K^+ exchanger of rod photoreceptors. The main intracellular loop mediates the effects of signals from the cytosolic side of the cell. It contains the XIP (eXchanger Inhibitory Peptide) domain, which has been described as a (pseudo)-calmodulin binding domain, Ca binding domain, which is responsible for the regulation of the exchanger by intracellular Ca^{2+} , alternative splicing site and a potential phosphorylation site.

The NCX1 gene is widely expressed in mammalian tissues. It is expressed at high level in the myocardium but can also be detected in several tissues (brain, kidney, smooth muscle, lung, skeletal muscle, pancreas, placenta) (Kofuji, et al., 1992; Blaustein, et al., 1991), although the mRNA levels are less than that seen in the myocardium. The isoforms of NCX1 that have been identified include: heart (NACA1), kidney (NACA 2, 3), brain (NACA4-6) (Kofuji et al., 1993a). These isoforms have a high degree of amino acid sequence homology (>90%). The only structural difference among the NCX1 isoforms is in a small region toward the end of the cytoplasmic loop, as a result of alternative splicing. The splicing variants are located in a region involved in regulation by Ca^{2+} and by inhibitory peptide interaction (Matsuoka, 1993) suggesting that the isoforms vary in their regulation.

Ret X (Na^+-Ca^{2+}, K^+ exchanger)

The Na^+-Ca^{2+}, K^+ exchanger was cloned from rod outer segments (RetX) in 1992 (Reilander et al., 1992). The hydropathy analysis suggests that the topology model of RetX is very similar to that of NCX1: 11 transmembrane domains and a large cytoplasmic loop that is responsible for regulation. However, RetX has little amino acid sequence homology with NCX1. The greatest regions of similarity between NCX1 and Ret X are in TM segments 2,3, 7 and 8. The rod exchanger is larger, containing 1199 AA and is heavily glycosylated with 6 consensus glycosylation sites in the amino-terminal domain. The rod type has a very restricted tissue distribution. Until now, its mRNA has not been detected by Northern blot analysis in any other tissues except the retina. It was reported

that K^+-dependent exchange activity exists in synaptosomal membranes, which suggest that nervous tissue may exhibit both K^+-dependent and K^+-independent exchangers (Dahan et al., 1991).

NCX 2 (the brain-type exchanger)

Another type of Na^+-Ca^{2+} exchanger, the NCX2 was cloned from rat brain in 1994 (Li et al., 1994). NCX2 transcripts can only be detected in brain and skeletal muscle by Northern blot analysis. The significance of this highly specific pattern of tissue expression for NCX2 in unknown. NCX2 has general similar functional properties to NCX1 and its amino acid sequence is 65% identical to NCX1. The NCX1 gene was located at the short arm of human chromosome 2, whereas the NCX2 gene was mapped to human chromosome 14.

IV. 3 MODULATION

Modulation relates to the processes that can modify the activity of the exchanger without necessarily changing the driving force of the transported ions. The regulation of the Na^+-Ca^{2+} exchanger has been most extensively studied in cardiac myocytes and squid axons. The Na^+-Ca^{2+} exchanger activity may be regulated by many extra- and intracellular factors such as ATP, intracellular Ca^{2+}(allosteric), phosphorylation, pH and calmodulin (XIP). It can also be regulated by kinases and hormones in transcription levels.

ATP and Intracellular calcium

Operation of any modes of the Na^+-Ca^{2+} exchanger has an absolute requirement for intracellular Ca^{2+} and is activated by intracellular ATP (Dipolo and Beauge, 1991).

The activation of the Na^+-Ca^{2+} exchanger by intracellular calcium was first observed from isotopic studies in squid axon (Allen and Baker, 1985; Dipolo and Beauge, 1987) and in dialyzed heart cells as a dependence of exchanger-mediated outward current on intracellular Ca (Kimura et al., 1986). It was shown that Ca^{2+} was needed to bind to a nontransport internal site of the exchanger and this was called the Ca^{2+} regulatory binding site (Hilgemann, 1990). This high affinity regulatory binding site is distinct from the Ca^{2+} transport site and is located in the middle of the cytoplasmic loop of the exchanger (Philipson et al., 1996) (see figure 1).

It has also been shown that ATP can stimulate reversed exchange and there seems to be a relationship (or interaction) between $Ca^{2+}{}_i$ and ATP. It was demonstrated that ATP acted by increasing the apparent affinity of Ca^{2+} for the intracellular Ca^{2+} regulatory site and the effect of ATP results from a phosphorylation process that involves the interplay of a coupled kinase(s)-phosphatase(s) system (Dipolo and Beauge, 1991).

XIP (eXchange Inhibitory Peptide)

XIP is a 20 amino acid peptide synthesized based on the molecular structure of the cardiac Na^+-Ca^{2+} exchanger. At the N-terminal side of the cytoplasmic loop of the cardiac Na^+-Ca^{2+} exchanger there is a positively charged region (219-238) rich in

arginine and lysine (see figure 1) (Nicoll et al., 1990). Because of its positive nature, it may represent a calmodulin binding site. It was found (Li et al., 1991) that the synthetic peptide corresponding to this 20 amino acid region can bind calmodulin and can inhibit Na^+-Ca^{2+} exchanger activity when applied from the inside of the cell (Chin et al., 1993). Therefore this synthesized 20 amino acid peptide is referred to as XIP for eXchange Inhibitory Peptide and that 20 amino acid region in the cardiac NCX corresponding to XIP is called the XIP domain. Mutants missing the XIP domain retain transport activity but lose the ability to be regulated by XIP (Khananshvili, 1994).

It has been suggested that XIP and the XIP domain of the Na^+-Ca^{2+} exchanger protein have a possible role as an autoregulatory (inhibitory) region (Philipson, 1993). As shown in figure 1, the endogenous XIP site is associated with the Na^+-dependent inactivation which is an intrinsic regulation for the Na^+-Ca^{2+} exchanger. The inactivation is thought to originate from a state of the exchanger with Na^+ ions bound at the intracellular surface. When Na^+ is applied to the intracellular surface, 3 Na^+ ions will bind at the intracellular transport sites. From this Na^+-bound state, the exchanger will transport Na^+ out of the cell across the membrane or it will enter an inactivated state. The distribution of the active and inactive states of the exchanger will determine the steady state level of outward exchange current (Philipson et al., 1996).

The secondary Ca^{2+} regulation and Na-dependent inactivation are not completely independent and the whole process is not fully understood. The inactivation process can

be altered by mutations in the endogenous XIP region. When a small deletion is made in this region, Ca^{2+} regulation is still present but Na^{+}-dependent inactivation disappears.

pH

Protons have a powerful regulatory role on the Na^{+}-Ca^{2+} exchanger, which was first reported in crab nerves (Baker and Blaustein, 1968). Baker et al., showed that extracellular alkalization from pH 6.0 to 8.5 increased Na^{+}-dependent Ca^{2+} efflux 3 fold, suggesting that protons could inhibit the Na^{+}-Ca^{2+} exchanger. In 1977 it was found (Baker and Mcnaughton,1977) that cytoplasmic acidification inhibited Ca^{2+}-dependent Na^{+} efflux in the giant squid axon. In 1982, Philipson et al., (Philipson et al., 1982) demonstrated that in canine cardiac sarcolemmal vesicles, changing pH from 6.0 to 10.0 increased Na^{+}-dependent Ca^{2+} uptake 15 fold. It was suggested that this observed effect of pH was mainly exerted from the cytoplasmic side of the membrane. Protons may modulate the cardiac Na^{+}-Ca^{2+} exchanger at multiple sites via multiple mechanisms, possibly at the Ca^{2+} or Na^{+} translocation site.

The modulatory effect of protons on the Na^{+}-Ca^{2+} exchanger may have important implications. Although the pH in the environment of the cell is tightly regulated, in some conditions it could be changed. For example, hypoxia could produce cytoplasmic acidification.

Phosphorylation

Information about regulation of the Na^+-Ca^{2+} exchanger by phosphorylation varies from tissue to tissue. In cardiac myocytes, the Na^+-Ca^{2+} exchanger can be regulated by both protein kinase A (Fan et al., 1996) and PKC (Iwamoto et al., 1996). For the squid giant axon Na^+-Ca^{2+} exchanger, there is much evidence suggesting that protein kinase-dependent phosphorylation is responsible for its stimulation by MgATP (Dipolo and Beauge, 1991).

The modulation of Na^+-Ca^{2+} exchanger by phosphorylation in smooth muscle has been very well studied. It has been reported that in vascular smooth muscle, the Na^+-Ca^{2+} exchanger is stimulated by phorbol esters, 8-bromo-cGMP, norepinephrine, high K^+ and platelet-derived growth factor (PDGF)-BB, suggesting that protein phosphorylation plays an important role in the activation of the exchanger. Recently, there has emerged direct evidence showing that the Na^+-Ca^{2+} exchanger of rat aortic VSMCs is phosphorylated at serine residues and concomitantly activated in response to physiological ligands such as PDGF-BB and alpha-thrombin or PMA (Shigekawa et al., 1996).

The Na^+-Ca^{2+} exchanger has been cloned from aorta (Nakasaki et al., 1993) and has been shown to have a very similar structure to that of the canine cardiac exchanger. It has been suggested that there may be three candidate serine residue-containing sequences in the cytoplasmic domain which can be phosphorylated by protein kinase C or Ca^{2+}/calmodulin kinase II. This sequence is also conserved in the cardiac exchanger (see figure 1).

Regulation of Expression

Studies concerning the regulation of Na^+-Ca^{2+} exchanger expression are very recent and are emerging. It has been shown that during cardiac development, the expression of Na^+-Ca^{2+} exchanger is maximal at birth and then decreases postnatally (Boerth et al., 1994). The factors that cause this normal postnatal downregulation of the Na^+-Ca^{2+} exchanger have not been characterized. One explanation is that because the immature mammalian heart is deficient in SR, it is thus relatively more dependent upon transsarcolemmal Ca^{2+} fluxes for regulating contraction and relaxation.

It has also been demonstrated that hormones, growth factors and protein kinases regulate Na^+-Ca^{2+} exchanger mRNA levels and activity and it seems that the regulation is stimulus and cell type specific (Smith et al., 1996). For example, activation of protein kinase C downregulated the Na^+-Ca^{2+} exchanger mRNA and activity in kidney epithelial cells, but had no effect on those of VSMC (Smith et al., 1995). In aortic myocytes, dexamethasone downregulated the Na^+-Ca^{2+} exchanger mRNA activity without effect on that of kidney epithelial cells (Smith, 1994). However the biochemical pathways by which protein kinases and external stimuli regulate Na^+-Ca^{2+} exchanger expression are unclear.

IV.4 INHIBITORS

In cells which have complex Ca^{2+} signaling systems with several different Ca^{2+} entry and removal pathways, it is important to have a specific inhibitor to separate the Na^+-Ca^{2+} exchanger from other pathways.

Amiloride Analogues

Currently, amiloride analogues are the most frequently used inhibitors of the Na^+-Ca^{2+} exchanger. The synthesis of amiloride and amiloride analogs was first described by Cragoe (Cragoe et al., 1967). Amiloride was initially demonstrated to inhibit the Na^+ channel present in renal epithelia. Amiloride and its analogs were then shown to "specifically" inhibit other ion transporters (Benos, 1982) such as Na^+-Ca^{2+} exchanger, Na^+-H^+ exchanger, Ca^{2+} channels, Na^+ pump, and K^+ channels (Kleyman and Cragoe, 1988). However the specificities are different. Based on their specificities, the amiloride analogs can be roughly divided into 3 groups: Na^+ channel blockers (i.e phenamil), Na^+-Ca^{2+} exchanger blockers (i.e CBDMB, (5-(4-chlorobenzyl)2'4'-dimethylbenzamil)) and Na^+-H^+ exchanger blockers (i.e. EIPA (5-(N-ethyl-N-isopropyl)-amiloride).

Amiloride is a pyrazinoylguanidine having amino groups on the 3- and 5-positions and a chloro group on the 6-position of the pyrazine ring. It is a poor inhibitor of the Na^+-Ca^{2+} exchanger (IC_{50}=1mM). DCB (3'.4'-dichlorobenzamil) has been used to inhibit the Na^+-Ca^{2+} exchanger (Harrison and Lancaster, 1994). However, it is not a highly specific inhibitor for the Na^+-Ca^{2+} exchanger (IC_{50}=29 uM) but is a specific inhibitor for Na^+ channels (IC_{50}=0.085 uM) (Kleyman and Cragoe, 1988). In addition, it may have effects on SR Ca^{2+} ATPase, the Na^+ pump, cAMP-dependent phosphodiesterase, voltage-gated Ca^{2+} channels and mitochondria oxidative phosphorylation in the same or lower concentration range as that which inhibits the Na^+-Ca^{2+} exchanger (Floreani et al., 1987; Bielefeld et al., 1986).

CBDMB (5-(4-chlorobenzyl)2'4'-dimethylbenzamil) has a similar structure to that of DCB, but has an aryl group on the pyrazine ring, conferring more specificity for the Na^+-Ca^{2+} exchange (IC_{50} is 7.3uM) (Kleyman and Cragoe, 1988). CBDMB has been tested in many different tissues and found to be a very potent and specific inhibitor of the Na^+-Ca^{2+} exchanger (Hunyady et al., 1988).

XIP

XIP is a more specific and potent Na^+-Ca^{2+} exchanger blocker than the amiloride analogues. However, in spite of the clear structural concept that led to its synthesis, the exact molecular inhibitory mechanism has not been clarified.

XIP has been successfully used in cardiac muscle and revealed that the Na^+-Ca^{2+} exchanger has physiological roles in cardiac Ca^{2+} signaling and has possible roles in EC coupling (Kohmoto et al., 1994). Nevertheless for most physiological experiments, it is difficult to use because the site of action for XIP is at the intracellular surface, thus it has to be applied into the cell in order to have an inhibitory effect.

IV.5 Physiological Functions

It is well established that the Na^+-Ca^{2+} exchanger is essential for Ca^{2+} homeostasis in many cells and is involved in physiological functions such as cardiac contractility, vision, secretion. The abundance of the Na^+-Ca^{2+} exchanger protein and its function vary in different cell types (Kofuji et al., 1992). In some cells, the Na^+-Ca^{2+} exchanger is the

primary Ca^{2+} extrusion mechanism, and small changes in its activity have large effects on cell function, whereas in other cells, it plays only a minor role in Ca^{2+} regulation.

Cardiac muscle

The sarcolemmal Na^{+}-Ca^{2+} exchanger is the major Ca^{2+} extrusion mechanism in cardiac muscle. When L-type Ca^{2+} channels are activated, Ca^{2+} influx via these channels increases local $[Ca^{2+}]_i$ sufficiently to activate the Ca^{2+}-release channels (ryanodine receptors) in the SR by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. These events take place at the transverse-tubule-ryanodine receptor junction. The Na^{+}-Ca^{2+} exchanger proteins are well placed to extrude the activating Ca^{2+} that enters the cell from the extracellular space, including the T-tubules. In cardiac muscle, there are about 250-400 Na^{+}-Ca^{2+} exchanger proteins per square micron, and this extrusion mechanism is very efficient. On the other hand, following depolarization and increase of local intracellular Na^{+}, the Na^{+}-Ca^{2+} exchanger could also mediate Ca^{2+} entry (operating in the reverse mode) (Leblanc and Hume, 1990; Lipp and Niggli, 1994). The location of the exchanger makes it possible to provide Ca^{2+} to activate ryanodine receptors in the SR.

Kidney

Heart, brain and kidney are the 3 richest sources of the Na^{+}-Ca^{2+} exchanger protein in mammalians (e.g., human) (Kofuji et al., 1992). The Na^{+}-Ca^{2+} exchanger was found to be located in the basolateral membrane of connecting tube epithelial cells in canine and rabbit kidney (Bourdeau et al., 1993; Kofuji et al., 1993b; Reilly, 1993). Presumably this

location indicates that the Na^+-Ca^{2+} exchanger is important in reabsorbing Ca^{2+} and controlling overall renal Ca^{2+} transport. Interestingly, different isoforms of the Na^+-Ca^{2+} exchanger are found in the kidney (Kofuji et al., 1993a) suggesting that they may have distinct functions in the kidney, but this has not been demonstrated.

Smooth Muscle Cells

The Na^+-Ca^{2+} exchanger was identified in VSMC by ^{45}Ca fluxes and contraction experiments (Reuter et al., 1973). Similar to many other cell types, in VSMC altering the extracellular Na^+ concentration or ouabain application produces changes in Ca^{2+} metabolism (Blaustein et al., 1991). Immunoblot, Northern blot and immunolocalization data suggest that the Na^+-Ca^{2+} exchanger in VSMC is the cardiac type but that the density is much lower. However, the contribution of Na^+-Ca^{2+} exchanger to overall Ca^{2+} homeostasis is not clear. One of the roles of the Na^+-Ca^{2+} exchanger could be in the indirect regulation of the intracellular Ca^{2+} stores in the SR.

IV.6 PATHOLOGICAL ROLES

Anoxia/ischemia

Anoxia/ischemia can cause irreversible damage to myelinated axons of the central nervous system. As in gray matter influx of extracellular Ca^{2+} plays an important role in the development of white matter injury. However the mechanisms of this damaging Ca^{2+} entry are different from those in neuronal cell bodies and synaptic terminals, because it is not dependent on excitotoxin- or voltage-gated Ca^{2+} channels. Recently it has been

shown that most of the deleterious Ca^{2+} influx in white matter is mediated by the reverse mode of the Na^{+}-Ca^{2+} exchanger (Stys and Steffensen, 1996). The hypothetical sequence of events in white matter injury induced by anoxia/ischemia is as follows: anoxia/ischemia produces cellular energy depletion and thus a decrease of Na^{+}-K^{+} ATPase activity, which increases intracellular Na^{+} concentration. The increase in $[Na^{+}]_i$ and membrane depolarization can both drive the Na^{+}-Ca^{2+} exchanger to the reverse mode (mediating Ca^{2+} influx) leading to intracellular Ca^{2+} overload. Excess Ca^{2+} then activates multiple Ca^{2+}-dependent biochemical systems and produces mitochondrial injury and damage to the structural integrity of the axon. Based on this hypothesis, Na^{+}-Ca^{2+} exchanger blockers and /or agents that can prevent or delay Na^{+} loading and depolarization may be used for therapies in diseases where white matter is damaged by anoxia/ischemia.

Cardiac hypertrophy and heart failure

It has been observed that Na^{+}-Ca^{2+} exchange activity is increased in animal models of cardiac hypertrophy and failure (Nakanishi et al., 1989). Recently, Studer et al., (1994) reported that in human failing myocardium, the mRNA and protein levels of the sarcolemmal Na^{+}-Ca^{2+} exchanger are increased whereas the SR Ca^{2+} pump mRNA levels are significantly reduced. This increased expression of the Na^{+}-Ca^{2+} exchanger is functionally important because increased Ca^{2+} extrusion by this mechanism can be a compensatory adaptation for the impaired SR pump in the failing heart therefore limiting intracellular Ca^{2+} overload.

PART V. GLUCOSE TOXICITY

V.1 GLUCOSE TOXICITY AND DIABETIC VASCULAR COMPLICATIONS

As described in the previous section, despite insulin treatment, most diabetic patients eventually develop one or more of the long-term complications of the disease (Nathan, 1993). Exactly what factors cause these complications have been debated for many years. Since almost all the vessels in the body can be affected in diabetes, it has been suggested that systemic factors play a major role in diabetic vascular complications.

It has been shown that classical risk factors for cardiovascular diseases are also operative in diabetic patients (Wingard et al., 1983; Jarrett et al., 1982). NIDDM and patients with impaired glucose tolerance are commonly obese and have hypertension and dyslipidemia (increased serum triglyceride and decreased high-density lipoprotein cholesterol concentrations). The imposition of hypertension, smoking or lipid abnormalities upon diabetes, exaggerates the incidence of cardiovascular disease and mortality (Stamler, 1987). However, in spite of these variables, diabetes itself remains an independent risk factor for coronary artery disease (Gordon, et al., 1977; Stamler, 1987). Recently the NIH-sponsored diabetes control and complications trial (DCCT) has been completed. The main conclusion reached from the results of this trial is that hyperglycemia is a major cause of diabetic vascular complications, which implies that glucose is a potentially toxic molecule (DCCT Research Group, 1993).

In addition to this trial, there are other clues suggesting that hyperglycemia can result in tissue damage. (a) In diabetes the metabolic abnormalities are necessary, if not sufficient, for the development of complications (Leslie and Pyke, 1982). (b) Among the many and diverse metabolic abnormalities of diabetes, the frequency and severity of complications correlate best with glucose levels or integrated indices of glycemia (Weat et al., 1980; Singer et al., 1992). (c) Diabetes and possibly high glucose can disturb fundamental cellular processes because it has been reported that diabetes is teratogenic. The risk of major malformations is markedly enhanced when first-trimester glycohemoglobin is high (Greene et al, 1989). (d)The glucose molecule has an intrinsic feature with potential disruptive consequences. For example, the aldehyde group of glucose is the substrate for the first reaction in the polyol pathway. Additionally in the open-chain form of the molecule, it is prone to transition metal-catalyzed auto-oxidation and nonenzymatic reaction with the amino groups of proteins (Pugliese et al., 1991). (e) Most of the above reactions of the glucose molecule and attendant metabolic imbalances have been shown to occur in diabetic tissues (Baynes, 1991; Greene et al., 1987).

On one hand, glucose is a molecule essential for life. Blood glucose is a precursor for the biosynthesis of glycogen and fat as well as various sugar-containing macromolecules, such as glycoproteins, glycolipids, and nucleic acids. Some tissues (e.g.brain) are almost entirely dependent upon glucose as an energy source, and other tissues (e.g. muscle) will preferentially catabolize glucose for ATP production when it is plentiful. On the other hand, the concentration of glucose must be carefully controlled

because too much glucose may have powerful adverse effects. Why glucose is potentially toxic has not been completely understood.

Because the sites of complications correspond to those tissues in which the glucose transport is insulin-insensitive, the intracellular metabolism of excess glucose may play an important role in the initiation and progression of the pathogenic sequence leading to long-term diabetic complications.

Based on the theory that platelets play an important role in the development of diabetic complications and on the evidence that glucose can be toxic, it is possible that hyperglycemia may be the factor which causes the platelet abnormalities in diabetes. Therefore, the objective of the last part of this study was to investigate the effect of glucose on platelets. Reviewed here, is a brief understanding of glucose transport and glucose metabolism in platelets.

V.2 GLUCOSE TRANSPORTERS

Since glucose is a hydrophilic molecule, it has to be transported initially across the hydrophobic core of the plasma membrane. Glucose transport is essential in light of its role in cellular homeostasis and metabolism. There are 2 kinds of glucose transporters. One is the simple facilitative glucose unitransporter and the other is the Na^+-dependent glucose cotransporter.

Facilitative Glucose Transporters

The facilitative glucose transporter exists in the plasma membrane of almost all cells. They transport glucose across the plasma membrane down its chemical gradient and mediate the exchange of glucose between the blood and the cytoplasm of the cell. They are specific for the D-enantiomer of glucose and are not coupled to any energy source such as ATP.

Until now, 6 mammalian glucose transporter isoforms have been identified as reviewed by James (1995). The human genes encoding these proteins are GLUT1 to 5 and GLUT7 and the corresponding proteins are Glut1, Glut2, etc. Each isoform consists of 12 transmembrane domains and the size varies from 492 to 524 amino acids. There is a high degree of homology among different isoforms but the tissue distribution, kinetic properties, and regulation are distinct, reflecting the unique glucose requirements of various tissues.

Glut1 is expressed at high levels in red blood cells, endothelial or epithelial-like barrier cells of the brain, eye, peripheral nerve and placenta (Takata et al., 1990). Its role is to provide glucose transport in various cells comprising a barrier between a body tissue and the blood supply. For example, the high expression of Glut1 in brain capillaries that comprise the blood-brain barrier, is of physiological importance, because brain parenchymal cells are completely dependent on blood glucose as an energy source in normal conditions. In addition Glut1 is expressed at low levels in some cell types together with some other tissue-restricted glucose transporter isoforms. For example, in insulin-

sensitive tissues (fat and muscle), Glut1 is present constitutively in the plasma membrane, providing the low level of glucose required for basal cellular activity. The tissue-specific Glut4 isoform is present in a special intracellular membrane compartment in the basal state and is translocated to the plasma membrane in response to insulin or other stimuli that signal the need for higher levels of cellular glucose transport. The K_m of Glut1 for glucose is 5-10 mM, thus it operates at close to its maximal transport velocity under physiological conditions.

Glut2 is found at high levels in the liver, small intestine, kidney and pancreatic beta-cells. In the kidney and the small intestine, Glut2 is localized to the basolateral membrane of epithelial cells, whereas the Na^+-dependent glucose cotransporter is in the apical surface. The Na^+-dependent glucose cotransporter concentrates glucose intracellularly within this epithelial layer, and then Glut2 transports glucose into the bloodstream. The K_m of Glut2 for glucose is 20-40 mM, thus it functions within the most linear part of its velocity-substrate curve under physiological conditions. This is physiologically important in hepatocyte and pancreatic beta-cells, because in response to glucose, the liver shuts off glucose output and pancreatic beta-cells secrete insulin.

Glut3 is the major glucose transporter in brain parenchymal cells. It is expressed at high levels in neurons, in the placenta and in the testes (Bell et al., 1993). The K_m of Glut3 for glucose is 1-5 mM which ensures that essential organs such as brain and placenta can obtain an adequate glucose supply even under conditions of starvation.

Glut4 is only expressed in those cells (adipose tissue, skeletal and cardiac muscles) in which the glucose transport is insulin-sensitive. The mechanism for the regulation of glucose transport involves the insulin-dependent Glut4 translocation. In the basal state, Glut4 is within the cell in small tubulovesicular elements that are associated with the trans-Golgi reticulum and endosomes. After insulin stimulation, Glut4 is translocated from intracellular vesicles to the plasma membrane. The role of Gut4 is to remove glucose from the bloodstream in the postprandial state and in whole-body glucose homeostasis.

Glut5 is a fructose transporter abundant in spermatozoa and in the apical membrane of intestinal cells. GLUT6 is a pseudogene that is not expressed at the protein level. Glut7 is the microsomal glucose transporter in liver (James, 1995).

In many cell types, glucose transport is not a rate-limiting step for overall cellular glucose metabolism and is therefore not subject to acute regulation. For example, GLUT1,2,3, the major glucose transporter isoforms expressed in endothelial cells, hepatocytes and neurons, respectively, are expressed constitutively at high levels in the plasma membrane. Glucose uptake in these cells depends primarily on the circulating glucose concentration and is largely unaffected by changes in the concentrations of hormones and other circulatory factors. On the other hand, in the so-called insulin-sensitive tissues such as muscle and fat, cellular glucose uptake increases dramatically within minutes in response to elevated blood insulin levels.

Na^+-Dependent Glucose Transporter

The Na^+-dependent glucose transport system is unrelated to the facilitative glucose transporters (Hediger et al., 1987). It is located in the apical membranes of epithelial cells in kidney and intestine and its function is to accumulate glucose. This glucose transport system is a secondary active transporter which actively pumps glucose from the intestinal or tubular lumen against its concentration gradient by coupling the transport of glucose and Na^+. The Na^+ gradient is maintained by the active transport of Na^+ across the basolateral surface of the brush border of the cells by the Na^+K^+ ATPase.

V.3 Glucose Transporter and Metabolism in Platelets

In platelets the major energy sources are glucose and glycogen (Akkerman, 1987). Fructose and mannose can be also used, but the rate of their use is less than 5% of that of glucose. Fatty acids can be oxidized by beta-oxidation (Akkerman and Verhoeven, 1987; Holmsen and Farstad, 1987). Whether amino acids can be used as effective energy sources in platelets is not clear, although platelets can be stored for days with glutamine as the only energy source without losing ATP or responsiveness to agonists.

Platelet Glucose Transporter

Platelets take up D-glucose from the medium by a stereoselective, saturable process that also transports 2-deoxyglucose and possibly fructose and mannose. This facilitative glucose transporter demonstrated high affinity for D-galactose and was

inhibited by cytochalasin B and forskolin. Hexose transport is not the rate-limiting step in glycolysis in resting and stimulated platelets (Holmsen and Farstad, 1987).

Recently, Craik et al., (1995) found that the glucose transporter in human platelets is the Glut3 (brain type) by using an immunoblotting method. On the immunoblot of platelet proteins the Glut-2 transporter isoform was not detected and only traces of the Glut-1 glucose transporter polypeptide were detected, which was explained as a contamination of red blood cells.

Platelet Glucose Metabolism

After glucose enters the platelet, it is phosphorylated by hexokinase to G-6-P. This effective phosphorylation step of glucose to G-6-P in platelets can create a steep glucose concentration gradient a cross the plasma membrane (outside>inside), which drives the uptake of glucose. G-6-P can be metabolized by 4 pathways: (1) glycolysis. (2) the hexose monophosphate shunt (HMPS). (3) glycogenesis and (4) dephosphorylation to glucose. Glucose-6-phosphatase has been demonstrated in platelet microsomes, but dephosphorylation probably plays a minor role in the intact cell and the conversion to glycogen is practically inoperative in isolated platelets. Therefore, in isolated platelets, the main directions of G-6-P metabolism are through glycolysis and HMPS (Holmsen and Farstad, 1987).

Platelets have a well-developed glycolytic system. Glycolysis supported by external glucose or by an intracellular glycogen store is the primary metabolic machinery

or regeneration of free energy (Akkerman, 1978). The glycolytic flux rate in platelets is 13 times higher than that in erythrocytes and 6 times higher than that in skeletal muscle (Karpatkin and Langer, 1968). The glycolytic flux can be activated severalfold under a variety of conditions of platelet stimulation. The circulating platelet contains 40 to 80μM of glycogen-bound glucose residues X 10^{-11} platelets (Holmsen and Farstad, 1987) and glycogen granules serve as an energy reserve for glycolysis (Akkerman et al., 1983).

Platelets have only a few mitochondria (6 to 8 per cell), each with a volume of 0.1 um^3. As a comparison, a mitochondrion from heart muscle is as big as a platelet, with a volume of about 7 um^3. Therefore, the metabolic energy in platelets is mainly derived from glycolysis rather than from oxidative phosphorylation (Holmsen, 1977; Chaudry et al., 1973).

The HMPS pathway is not energy-yielding, but it is important for production of NADH and can be a major route for G-6-P degradation.

V.4 MECHANISMS OF GLUCOSE TOXICITY

Because glucose and its metabolites are utilized by numerous intracellular pathways, the adverse effects of hyperglycemia involve multiple mechanisms. Until now, 4 theories have been postulated to link hyperglycemia and vascular complications: (a) the sorbitol hypothesis; (b) nonenzymatic glycation; (c) redox potential alteration and (d) the diacylglycerol-protein kinase C pathway.

The Sorbitol Hypothesis

In most cells, glucose can be converted to sorbitol by an aldose reductase. When the glucose level is not elevated, intracellular sorbitol concentration is very low because the K_m of aldose reductase for glucose is high. When the intracellular glucose level increases, the intracellular level of sorbitol will increase. Once formed, sorbitol can be metabolized by sorbitol dehydrogenase into fructose, with the production of NADH. However, this degradation of sorbitol progresses relatively slowly, resulting in the accumulation of sorbitol in the vascular, neuronal, and other tissues (Greene et al., 1987; Kador et al., 1985). Because sorbitol does not diffuse across cell membranes easily, in some cells it may accumulate in the mM range and damage the cell by osmotic stress as demonstrated by the development of cataracts in diabetic patients (Kinoshita et al., 1981). In other cells (such as neural tissues), the sorbitol increases may not be high enough to cause significant osmotic changes, and may affect cell function by other mechanisms such as depletion of myoinositol (Pugliese et al., 1991).

Aldose reductase inhibitors have been under clinical testing for 20 years (Judzewitsch et al., 1983), but they remain to be proven effective enough to for Food and Drug Administration approval (Pfeifer and Schumer, 1995).

Nonenzymatic Glycation

Nonenzymatic glycation is a ketoamine reaction between glucose and the N-terminal amino acid of proteins. Further reactions involving these intermediate glycosylated compounds can lead to the formation of advanced glycosylation end products

(AGE) in an irreversible chemical reaction(Brownlee, 1994). The major factors that govern the rate of formation of these glycosylated products are the level of glucose and duration of exposure to glucose. It has been shown that nonenzymatic glycation can alter the function of many proteins and was involved in the pathogenesis of diabetic vascular complications. For example, glycated hemoglobin has an increased affinity for O_2 (Ditzel, 1976); glycated spectrin may be responsible for the reduced erythrocyte deformability in diabetes (McMillan and Brooks, 1982); glycated LDL lose affinity for their receptor (Witztum et al., 1982); and the altered physical properties of lens crystallin by glycation may be involved in cataract formation in diabetics (Stevens et al., 1978).

Chronic treatment with non-enzymatic glycation inhibitors such as aminoguanidine, which selectively blocks the reactive carbonyl group on early glycosylation products may reduce basement membrane thickening and prevent some of the early changes in the retinal vasculature of diabetic rats (Hammes et al, 1991). However the final conclusion will come with the clinical trial of aminoguanidine, which is currently in progress.

Redox Potential Alterations

This hypothesis postulates that the ratio of NADH/NAD is increased via the metabolism of glucose by glycolysis or through the polyol production pathway. The increased NADH/NAD ratio can then affect many other pathways such as DAG synthesis, DNA repair and fatty acid oxidation (Williamson et al., 1993).

Diacylglycerol-Protein Kinase C Pathway

In 1989, Lee et al., first reported that hyperglycemia in vitro can cause a persistent activation of protein kinase C (PKC) via an increase of diacylglycerol (DAG) levels in cultured vascular cells. Many studies subsequently showed that hyperglycemia increased DAG and PKC in many tissues such as retinal, aortic and mesangial cells (Inoguchi et al., 1992). In diabetic animals, PKC activity and total DAG levels have been found to be increased in VSMC and endothelial cells (Xia et al., 1994; Kaiser et al., 1992) suggesting that diabetes increases PKC activity by first increasing total DAG content of the tissue (Williams, 1995). The increased level of DAG results from the enhanced de-novo synthesis from glucose via the glycolytic pathway, as demonstrated by the increased [^{14}C]glucose incorporation into DAG (Lee et al., 1989). In addition, DAG levels are increased in the hearts of diabetic animals (Okumura et al., 1988). It appears that hyperglycemia-induced activation of PKC by de novo synthesis of DAG could be generalized to all vascular tissues involved in diabetic complications.

Since PKC is a serine/threonine protein kinase involved in the signal transduction and many cellular responses, the activation of DAG and PKC by hyperglycemia have multiple cellular consequences such as abnormalities of permeability, flow, contractility, coagulation, basement membrane thickening, cell growth and neovascularization (Nishio et al., 1994; Shiba et al., 1993). There are 12 isoforms of PKC. In vascular tissues, the beta-2 isoform is the key step in high glucose-induced activation of PKC and this activation has pathological significance.

PART VI. RATIONALE AND HYPOTHESES

Accelerated atherosclerosis and enhanced thrombosis as well as diabetic microangiopathy are characteristic of the diabetic state. These vascular diseases are the frequent cause of morbidity and mortality among patients with diabetes mellitus. Nevertheless, until now, the pathogenesis of both macroangiopathy and microangiopathy has not been completely understood. The overall objective of this thesis is to determine the mechanisms of platelet hyperactivity in diabetes.

It has been well established that platelets play important roles in accelerated atherosclerosis and microangiopathy. Platelet hyperactivity has been shown in vitro and in vivo in platelets from both type I and type II diabetic patients and in experimental diabetic animals. Since platelet hyperactivity can also be observed in washed platelets, the platelets per se may be responsible for the hyperactivity in diabetes mellitus. However the exact mechanism underlying platelet hyperactivity is not clear.

Platelet activation is a very complex process. There are many steps from receptor activation to a functional response (aggregation). Theoretically all these steps could be abnormal in diabetes.

In the literature, there is only minor evidence about abnormalities in platelet receptors or in the terminal functional systems. It seems more likely that the signal transduction mechanisms of platelets may be abnormal, which could, in turn, be responsible for platelet hyperactivity. The arachidonate pathway has been consistently

shown to be enhanced in both types of diabetes and there is a significant correlation between TXA_2 and glucose or HbA_{1c} levels. Based on this observation, it was expected that aspirin should have beneficial outcomes in diabetic patients. However, the results of clinical trials were not as expected. In vitro experiments also showed that in diabetes platelet hyperactivity to thrombin was independent of the activation of the arachidonate pathway. All this evidence suggests that in addition to the arachidonate pathway, other mechanisms are also involved in platelet hyperactivity in diabetes.

Cytosolic calcium is a critical second messenger in all platelet responses, its regulation is reported to be disturbed in several tissues of diabetic animals. Therefore, it was first hypothesized that the handling of cytosolic calcium was affected by diabetes mellitus which would result in platelet hyperactivity. This hypothesis was tested by comparing the resting calcium concentration and agonist stimulated cytosolic calcium transients between a group of diabetic patients and a group of nondiabetic subjects. Once it was determined that cytosolic calcium was increased in platelets from diabetes, a series of experiments was performed to determine the mechanism by which cytosolic calcium concentration was enhanced in diabetes.

In the literature, there was some evidence for an impaired exchange of calcium across the platelet plasma membrane in diabetes. Bergh (1988) studied calcium exchange into and out of the cytosol in platelets from 8 patients with insulin-treated diabetes. They found that the influx rate of ^{45}Ca was significantly increased in diabetic platelets compared with controls. The efflux rate was significantly reduced in diabetic patients. However the

mechanisms underlying this impaired calcium exchange in diabetes are not known. We focused on the Na^+-Ca^{2+} exchanger because it is a carrier mechanism which can mediate both Ca^{2+} efflux (forward mode) and Ca^{2+} influx (reverse mode). The direction and activity of the Na^+-Ca^{2+} exchanger is determined by the concentrations of Na^+ and Ca^{2+} as well as by membrane potential. In the diabetic state, these factors could be abnormal.

The second hypothesis of our project was that the activity and direction of the platelet Na^+-Ca^{2+} exchanger may be changed in diabetes which may, in turn, produce platelet cytosolic calcium increases and hyperactivity. We first determined the physiological role of the Na^+-Ca^{2+} exchanger in platelets using several different approaches. We then tested the pathological role of this exchanger.

After determining the abnormalities in platelet calcium homeostasis, the next part of this study was to determine what causes platelet calcium abnormality and platelet hyperactivity in diabetes. Since many studies, especially the large clinical trial (The diabetes control and complications trials research group, 1993) has provided conclusive evidence that hyperglycemia is correlated with the degree and extent of diabetic vascular complications, the third hypothesis was that hyperglycemia may be the factor which causes platelet calcium abnormality and hyperactivity. This hypothesis was tested by studying the direct effect of hyperglycemia on platelets from normal subjects.

MATERIALS AND METHODS

SUBJECTS

The diabetic patients were obtained from Dr. V. Woo's diabetic clinic at the Health Sciences Center, Winnipeg General Hospital, Manitoba, Canada. Most of these patients are type II diabetics with or without vascular complications. Some of these patients were under treatment with insulin and oral hypoglycemics. The glycosylated haemoglobin level (HbA_{1c}) was used as an index of metabolic control. Only patients who had poor metabolic control were selected. Glycohemoglobin is produced by a ketoamine reaction between glucose and the N-terminal amino acid of both beta chains of the hemoglobin molecule. The major form of glycohemoglobin is hemoglobin A_{1c}, which normally comprises only 4-6% of total hemoglobin. The remaining glycohemoglobins (2-4% of total hemoglobin) contain phosphorylated glucose or fructose and are termed hemoglobin A_{1a} and A_{1b}, respectively. The hemoglobin A_{1c} fraction is abnormally elevated in diabetics with chronic hyperglycemia and appears to correlate positively with metabolic control. The glycosylation of hemoglobin is dependent on the concentration of blood glucose. The reaction is not reversible, so that the half life of glycosylated hemoglobin relates to the life span of red cells (which normally circulate for up to 120 days). Thus

glycohemoglobin generally reflects the state of glycemia over the preceding 8-12 weeks, thus providing a method of assessing chronic diabetic control. A glycosylated Hb close to the normal range would reflect good control during the preceding 2-3 months, whereas an increased glycosylated Hb would reflect poor control during the same period.

HbA_{1c} was measured in the Department of Clinical Chemistry in the Health Science Center. The glyc-affin glycated hemoglobin system was used to separate the glycated hemoglobin from other hemoglobins as hemolysate passes through a pre-filled column. The resin in the column is an affinity medium composed of boronate groups bound to agarose. Glycohemoglobin binds to these groups as the sample travels through the column. The binding is based on the special affinity of boronate groups for the Cis-diol group of glucose. The non-glycated hemoglobin will not bind and is eluted in the first fraction. Glycated hemoglobin is eluted from the column with a second buffer which competes with protein bound glucose for the binding sites on the column. Absorbances at 415 nm of both fractions are corrected for elution volumes and the percent glycated hemoglobin is calculated. The normal range of HbA_{1c} is 3.4 to 5.4%.

The mean HbA_{1c} in our selected diabetics was >11%. The mean serum total triglyceride, cholesterol level, mean systolic blood pressure and diastolic blood pressure were recorded for these patients and were not significantly different from normal. The control subjects were normal healthy people. Their HbA_{1c} levels were in the normal range. None of the participants had taken any anti-platelet drugs in the previous 2 weeks.

PLATELET ISOLATION

Venous blood sample was drawn into 2 types of vacutainer tubes depending on the type of experiments. In most cases, the blood was drawn into yellow-capped tubes which contain sodium dextrose to prevent platelet aggregation and coagulation. When studying the effect of glucose on normal platelets, purple-capped tubes were used. The purple-capped tubes contain EDTA as an anticoagulant. Platelet rich plasma (PRP) was obtained by centrifugation at 600 rpm for 15 mins at 18^0C. Then the PRP was centrifugated at 2000rpm for 15minutes at 18°C . The supernatant (platelet-poor plasma) was aspirated and the sedimented pellets were resuspended in 500 µL of plasma and then loaded with fluorescent dyes.

MEASUREMENT OF PLATELET CYTOSOLIC FREE CALCIUM CONCENTRATION

Platelet intracellular free calcium concentration was quantitated by the Ca^{2+} sensitive fluorescent indicators. In most experiments, fura-2 was used. If 5-(4-chlorobenzyl-)-2',4'-dimethylbenzamil (CBDMB) was used in the experiments then a combination of Ca-green and fura-red was used, because CBDMB quenches fura-2 (Kraut et al., 1993).

SAMPLE LOADING

Fura-2 and calcium green/fura-red do not permeate cell membranes. The acetoxy methyl ester (AM) form of these dyes: fura-2 (10μM final concentration) or a combination of calcium-green (10 μM/L) and fura-red (20 μM/L) were added to the platelet suspension and incubated at 37°C for 30 minutes to 1 hour. This period of incubation is adequate for permeation of the AM form of the dye and hydrolysis of the ester so that the dye remains in the cytosol of the platelets. After incubation, the platelets were separated from plasma and extracellular dye by gel filtration using a Sepharose CL-2B column equilibrated first with a 1:9 dilution of 1.8% sodium citrate, 1% glucose solution and then equilibrated with calcium-free HEPES buffer. The eluted platelets were counted in a Coulter counter and adjusted to $2X10^8$/mL with the calcium-free HEPES buffer containing 140mM NaCl, 4.9mM KCl, 1.2 mM $MgCl_2$, 1.4mM KH_2PO_4, 11mM glucose, and 20mM HEPES (pH 7.4).

FLUORESCENCE MEASUREMENT

Fluorescence of fura-2 loaded platelets was measured in a Jasco Model CAF 100 intracellular Ca^{2+} ion analyzer which is equipped (described in Sato et al., 1988) with a Peltier thermoelectric control and a magnetic stirrer. A xenon high-pressure lamp (75 W) with a rotating filter wheel (48 Hz) with 340- and 380-nm (5.5 nm) interference filters was the source of the excitation light which shone into the round glass cuvettes containing fura-2-labelled cells. Emitted light that passed through a 500-nm (10 nm) filter was detected by a photomultiplier tube set at 270 V. Output of fluorescence intensity could be

monitored for each excitation wavelength (F340 or F380) and the ratio of fura-2 fluorescence emission at 500 nm produced by excitation at 340 and 380 nm (R340/R380). The R340/380 was recorded continually and the F340 or F380 readings were taken occasionally.

CALCULATIONS OF INTRACELLULAR FREE CALCIUM CONCENTRATION

Intracellular free calcium concentration was calculated from the formula: $[Ca^{2+}]_i = K_d \times [R-R_{min}]/[R_{max}-R] \times S_{f2}/S_{b2}$ as described by Grynkiewicz et al., (1985), where R is the ratio of fluorescence at 340 and 380 nm, and Rmax and Rmin are the ratios for fura-2 free acid fluorescence measured at the 2 wavelengths in the presence of saturating Ca^{2+} and in the presence of EGTA, respectively. K_d, the dissociation constant of fura-2, was considered to be 224 nM at 37°C. S_{f2} and S_{b2} are fluorescence intensities at 380 nm without Ca^{2+} and with saturating levels of Ca^{2+}, respectively. Rmax and Sb2 were measured after cellular lysis with 20 µM/L digitonin in the presence of 1 mM Ca^{2+}. Rmin and Sf2 were determined after lysis and addition of 10 mM EGTA. Autofluorescence was measured and found not to contribute more than 10% of the fura-2 signal.

For Ca-green-1/AM and fura-red/AM co-loaded platelets, the fluorescence was measured in a Jasco-110 analyzer. One excitation wavelength (500 nm) and dual emission wavelengths of 540 nm and 680 nm were used. When cytosolic Ca^{2+} concentrations increase, at 500 nm excitation wavelength, the emission intensity of calcium-green at 540 nm increases, whereas the emission intensity of fura-red at 680 nm decreases. By taking the ratio of emission intensity at 540 nm to 680 nm, intracellular free Ca^{2+} concentrations

can be estimated by the same formula as that for fura-2. The only difference is the K_d which was taken as 189 nM.

MEASUREMENT OF PLATELET AGGREGATION

Platelet aggregation was simultaneously measured with fluorescence conducted in the Jasco CAF-100 or 110 Ca^{2+} analyzer and the pattern was recorded. A stirrer speed of 1000 rpm was used and the temperature was set at 37^{0}C . Platelet aggregation was measured by optical density. As platelets aggregate, the number of total particles decreases, and the optical density of the suspension decreases. For the initiation of aggregation, thrombin (0.5U/mL-1.25U/mL) or collagen (2μg-20μg/mL) were used. The aggregation extent was determined as the change in optical transmission 1 and 3 minutes after the addition of agonists.

EXPERIMENTAL PROTOCOLS

RESTING $[Ca^{2+}]_i$ AND AGONIST-EVOKED $[Ca^{2+}]_i$ RESPONSE

Before measuring fluorescence the platelet sample was stored on ice (4^{0}C) to prevent dye leakage. When the measurement started, 500 μl of platelet suspension ($2X10^8$/mL) was placed into the cuvette, then 1mM external $CaCl_2$ was added. The platelets were allowed to equilibrate for 3 minutes then an agonist, such as thrombin, was added. The thrombin-evoked cytosolic transient $[Ca^{2+}]_i$ increase and the post-transient

$[Ca^{2+}]_i$ was monitored for a total of 5 minutes. The following variables were recorded (figure 2): (1). Platelet $[Ca^{2+}]_i$ before addition of thrombin, this was taken as the resting cytosolic $[Ca^{2+}]_i$ level. (2). The peak of thrombin-evoked cytosolic calcium increase, this value subtracting the basal $[Ca^{2+}]_i$ was taken as the peak 1 thrombin-induced $[Ca^{2+}]_i$ response. (3). The $[Ca^{2+}]_i$ at 1 minute and 3 minutes after the peak. The 1 min $[Ca^{2+}]_i$ value subtracting basal $[Ca^{2+}]_i$ was used as the thrombin-induced phase 2 response.

ESTIMATION OF THE SIZE OF INTRACELLULAR RELEASABLE CA^{2+} STORE

Similarly, 500 uL platelet suspension was placed in the cuvette, 1mM $CaCl_2$ was added then 5mM EGTA was added to chelate extracellular Ca^{2+}. 1 minute later, a maximal dose of ionomycin (5μM) was applied. In this condition, the external Ca^{2+} concentration was less than 10^{-8}M. The peak cytosolic Ca^{2+} response to 5 μM ionomycin indicates the total amount of releasable free Ca^{2+} in the intracellular storage sites such as DTS as described by Oshima et al (1991).

AGONIST RELEASABLE CA^{2+} AND THE RECOVERY OF CYTOSOLIC CA^{2+} AFTER STIMULATION

In the presence of EGTA, if platelets were triggered with thrombin, the peak of the thrombin-induced cytosolic Ca^{2+} response indicates thrombin-releasable Ca^{2+}. The thrombin-releasable pool is part of the total intracellular releasable Ca^{2+} store. To assess the recovery from the peak $[Ca^{2+}]_i$ response, the $[Ca^{2+}]_i$ was recorded for 5 minutes. The $[Ca^{2+}]_i$ response at 1minute after the peak was determined. If this $[Ca^{2+}]_i$ was higher, it

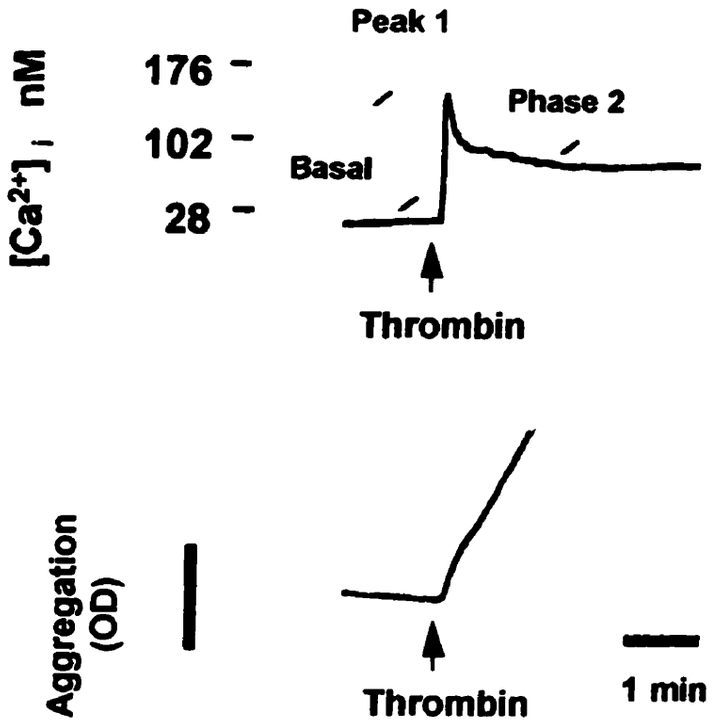


Figure 2

A typical trace showing the evaluation of resting platelet cytosolic free calcium concentration ($[Ca^{2+}]_i$) and thrombin-evoked $[Ca^{2+}]_i$ response. Fluorescent dye (here is Ca-green fura-red) loaded platelets were suspended in zero Ca^{2+} HEPES buffer. Then 500 µL of platelet suspension was put into the cuvette and 1 mM external Ca^{2+} was added. 3 minutes later thrombin (0.5 U/ml) was added. The $[Ca^{2+}]_i$ before the addition of thrombin was calculated as the basal $[Ca^{2+}]_i$. Peak 1 indicates the transient $[Ca^{2+}]_i$ rise after thrombin. Phase 2 indicates the sustained $[Ca^{2+}]_i$ rise after thrombin. When calculating these responses, basal $[Ca^{2+}]_i$ was subtracted.

indicate that after agonist stimulation the rate of removal of Ca^{2+} from the cytosolic compartment was slower.

ESTIMATION OF Na^+-Ca^{2+} EXCHANGER IN INTACT PLATELETS

Effect of reducing transmembrane Na^+ gradient

Partial replacement of external Na^+ with isomolar N-methyl-D-glucamine on Ca^{2+} homeostasis in intact human platelets: This protocol was designed to determine whether decreasing extracellular Na^+ concentration by partially substituting Na^+ with NMDG will alter Ca^{2+} homeostasis. While platelets were kept in Na^+-free medium, $[Na^+]_i$ was expected to fall to a very low level, eliminating the Na^+ gradient across the cell membrane. Therefore, the low Na^+ buffer (28 mM) was selected because in 28 mM extracellular Na^+ platelet $[Na^+]_i$ did not decline (Oshima, et al., 1994). A part of the platelet suspension in standard buffer and that in Na^+-free buffer were mixed to adjust the extracellular Na^+ concentration to 28 mM (1 volume of standard buffer and 4 volumes of NMDG buffer were used).

Sensitivity to Na^+-Ca^{2+} exchanger blocker CBDMB

The direction and activity of the Na^+-Ca^{2+} exchanger was studied indirectly in intact platelets by comparing the $[Ca^{2+}]_i$ before and after the blockade of the exchanger by an amiloride analogue CBDMB. As shown in the model of figure 3, if the function of the exchanger is to mediate Ca^{2+} efflux (forward mode), then after CBDMB the $[Ca^{2+}]_i$

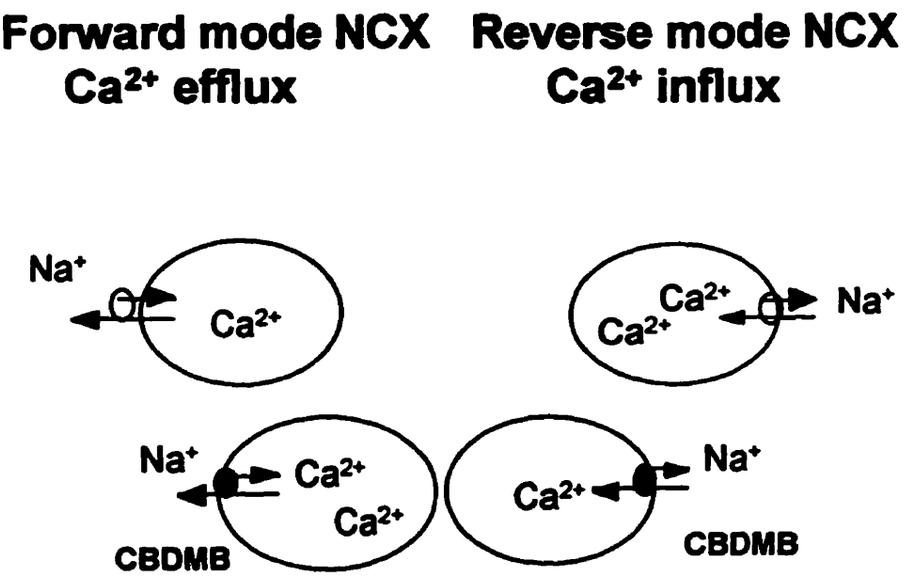


Figure 3

A model showing how to determine the direction of Na^+-Ca^{2+} exchanger in intact platelets by the sensitivity to CBDMB. The platelet $[Ca^{2+}]_i$ change after inhibiting Na^+-Ca^{2+}-exchanger by CBDMB was used as the sensitivity to CBDMB. If Na^+-Ca^{2+}-exchanger works in the forward mode (mediating Ca^{2+} efflux), then after inhibition by CBDMB the platelet $[Ca^{2+}]_i$ should increase. If the exchanger works in reverse mode (mediating Ca^{2+} influx), then after CBDMB the $[Ca^{2+}]_i$ should decrease.

should increase. If the role of the exchanger is to mediate calcium influx (reverse mode), then after CBDMB the $[Ca^{2+}]_i$ should decrease.

Na^+-dependent $[Ca^{2+}]_i$ decline in intact platelets

The forward mode of the Na^+-Ca^{2+} exchanger can be estimated by measuring extracellular Na^+-dependent $[Ca^{2+}]_i$ decline in intact platelets. First, the loaded platelets were suspended in 0 Na^+, 1 mM Calcium medium for 15 minutes. When the cytosolic $[Ca^{2+}]_i$ reaches a steady state, different concentrations of NaCl were added extracellularly. This extracellular addition of NaCl can produce a decrease in cytosolic $[Ca^{2+}]_i$. This Na^+-dependent $[Ca^{2+}]_i$ decline was taken as a measure of the forward mode of Na^+-Ca^{2+} exchanger (Na^+-dependent Ca^{2+} efflux).

THE DIRECT EFFECT OF HYPERGLYCEMIA ON PLATELET $[CA^{2+}]_i$ AND AGGREGATION:

Venous blood was drawn into EDTA containing vacutainers. PRP was then isolated by centrifugation and the glucose concentration was measured by the glucose oxidase method as described in the YSI Model 27 industrial analyzer manual. The average glucose level in the PRP of normal volunteers was 5 mM. The PRP from the same subject was divided into 3 portions: Control (no added extra Glucose), High glucose (40 mM glucose was added), High mannitol (40 mM mannitol was added to be used as an osmotic control). All the 3 portions of PRP were then incubated at 37°C for 24 hr. During incubation, the glucose concentration was monitored and additional glucose was added to balance the consumed glucose.

MEASUREMENT OF INTRACELLULAR PH

The procedures were similar to those of $[Ca^{2+}]_i$ determination except that platelets were incubated with 6 μM BCECF in a platelet suspension. The excitation wavelengths used were 500 and 440 nm, while the emission wavelength was at 540 nm. The fluorescence signal of BCECF was calibrated at the end of every experiment. This was done by adding KCl (80 mM) and nigericin (20 μM) to collapse the proton gradients, thereby making $[pH]_i$ equal to $[pH]_o$. A glass pH microelectrode was inserted into the cuvette to measure the pH at various times (Patel et al., 1991). A standard curve with different pH standards was used to calculate the $[pH]_i$ (Yu et al., 1991).

GLUCOSE TRANSPORT IN HUMAN PLATELETS

Glucose transport in platelets was measured by the influx of ^{14}C-labeled 3-O-methyl-D-glucose (3OMG). We chose 3-O-methyl-D-glucose (3OMG) because it has been used as a probe for glucose transport (Csaky and Wilson, 1956) for many years and it has several useful characteristics: (1) the kinetic constants of 3OMG are similar to those of glucose, as shown in frog skeletal muscle (Narahara and Ozand,1963) and isolated perfused rat heart (Morgan and Park, 1958); and (2) 3OMG is a nonmetabolizable glucose analogue so metabolic breakdown does not interfere with calculations of transport rates. Glucose transport was calculated by measuring the platelet/medium distribution of 3OMG.

The method was modified from the procedure described by Kim et al., (1986). Platelet rich plasma was obtained from the platelet concentrate. Platelet concentrate was prepared from fresh whole blood (random donor) supplied by the Canadian Red Cross Society Blood Services. Platelet concentrate contains approximately 60 X 10^9 platelets in 50-60 mL of plasma. The platelet concentrate was centrifuged at 2000 rpm for 15 minutes to isolate the platelet pellets. The platelet pellets were then washed with 1:9 citrate buffer first then with Ca^{2+}-free and Glucose-free buffer by centrifugation. The washed platelets were suspended to 2x10^9 platelets/mL in the Ca^{2+}-free and glucose free buffer. Uptake of radiolabeled 3OMG and unlabeled substrate were carried out at 37^{0}C.

Uptake was initiated by the addition of 0.4 mL of platelet suspension to an Eppendorf microcentrifuge tube containing 0.05 mL of Ca^{2+}-free and Glucose-free buffer with 1.0 μCi of [^{14}C]3OMG per mL and 0.6 mM unlabeled 3OMG at 37^{0}C. During the incubation the tubes were vortexed to achieve adequate mixing. Each uptake point was then terminated at the appropriate time by the addition of 1 mL of cold stopping solution which contains 2 mM $HgCl_2$ and 154 mM NaCl. Zero time was routinely determined by adding the stopping solution to the platelets before the addition of labeled substrate. Cells were quickly pelleted by centrifugation for 20 sec at 25^{0}C. The supernatant was aspirated and the cells were washed two times with 1 mL of cold stopping solution. Radioactivity was extracted from cells by lysing the pellet with 100 μl trichloroacetic acid (TCA). After thorough vortexing, liquid scintillation fluid was added and the radioactivity was measured. Each point is an average value of three replicates. The Ca^{2+}-free and Glucose-free buffer consisted of 140mM Na Cl, 2.5 mM KCl, 1 mM

KH_2PO_4 and 5 mM HEPES, pH 7.2. The appearance of radioactivity in the platelets was measured as a function of time, and the effect of insulin on 3OMG uptake was determined at 2 time points. Results were expressed as μM glucose/mL platelets.

CHEMICALS

Amiloride analogues, 5-(4-chlrobenzyl)-2',4'-dimethylbenzamil (CBDMB) and EIPA were purchased from E.J. Cragoe, Jr (P.O. Box 631548, Nacogdoches, TX 75963-1548). They were synthesized by previously described methods (Bicking et al., 1965; Cragoe et al., 1967; Jones et al., 1969; Cragoe et al., 1987). These compounds were dissolved in dimethyl sulfoxide (DMSO) to give stock solutions of 10 mM or 1 mM. Fura-2/AM, Calcium Green-1/AM and Fura red/AM are from Molecular Probes Inc, Eugene, OR, USA. They were kept as 1 mM stock solutions in DMSO. Thrombin (from bovine plasma) was obtained from Sigma Chemical Co. and was made up with water as a stock of 50 units/mL. Collagen was purchased from Nycomed Arzneimittel, the stock solution was 1 mg/mL. Sepharose 2B-CL was obtained from Pharmacia Biotechnology. All other chemicals (BSA, EGTA, NMDG chloride, sucrose) were of the highest analytical quality available and were purchased from Sigma.

STATISTICAL ANALYSIS

All the data were expressed as means±SE, n=number of subjects from whom platelets were obtained. Differences between means of data from nondiabetic subjects and diabetic patients were tested for significance using two-tailed Student t-tests for unpaired data. When comparisons were done within the same group between control and drug treatment, paired t-tests were used. In the figures statistically significant differences are indicated by * ($P<0.05$).

RESULTS

PART I: PLATELET CALCIUM HOMEOSTASIS IN DIABETES

RESTING PLATELET $[Ca^{2+}]_i$ LEVEL AND AGONIST-STIMULATED $[Ca^{2+}]_i$ RESPONSE

The resting $[Ca^{2+}]_i$ level of platelets from nondiabetic subjects was in the range of 80-100 nM. In platelets from diabetics patients resting $[Ca^{2+}]_i$ was higher (figure 4). In the presence of 1mM extracellular Ca^{2+}, addition of thrombin (0.5 U/ml and 1.25U/ml) increased $[Ca^{2+}]_i$ in platelets from both study groups. The typical thrombin $[Ca^{2+}]_i$ response can be divided into 2 phases: a rapid immediate $[Ca^{2+}]_i$ increase after thrombin addition (peak 1) and a sustained $[Ca^{2+}]_i$ level (phase 2) (figure 2). As shown in figure 5 there were no significant differences in the peak 1 $[Ca^{2+}]_i$ response between platelets from diabetic and nondiabetic groups when the platelets were stimulated by 0.5U/mL thrombin. However, the $[Ca^{2+}]_i$ level at 1 minute and 3 minutes after thrombin addition were significantly higher in platelets from diabetic patients compared to those of nondiabetic subjects. The same results were obtained when 1.25U/ml of thrombin was used (data not shown). Stimulation of platelets by collagen also produced a rise of $[Ca^{2+}]_i$ in both diabetics and controls. Figure 6 shows collagen increases $[Ca^{2+}]_i$ dose-dependently (from

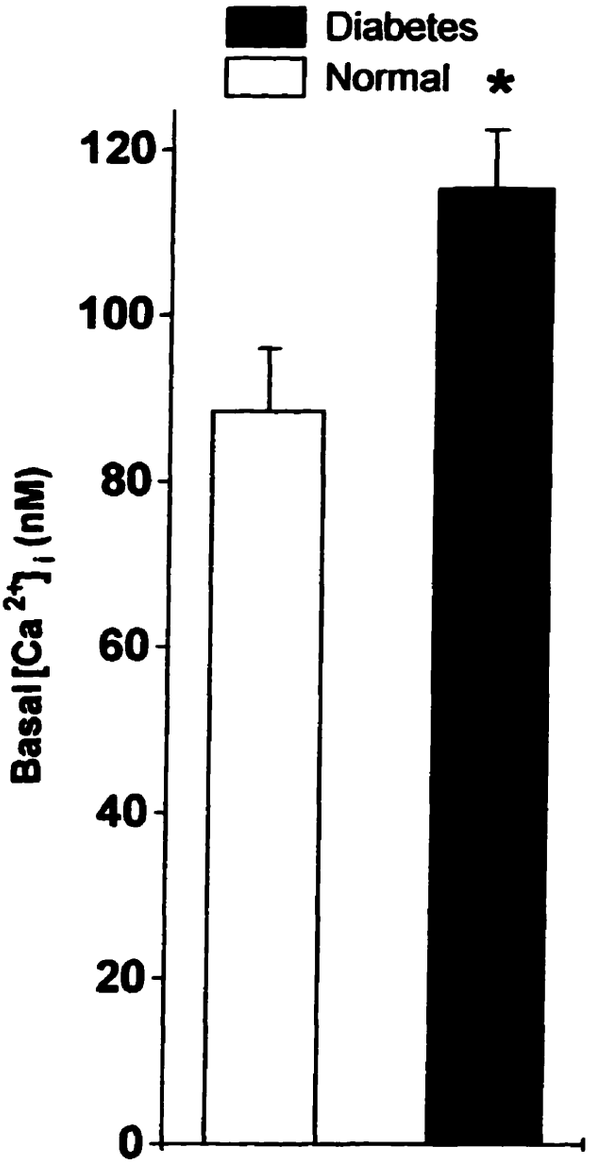


Figure 4

Resting $[Ca^{2+}]_i$ in platelets from a group of uncontrolled diabetic patients (n=15,black bar) and nondiabetic subjects (n=18,open bar). Platelets were loaded with fura-2 AM. Values are means ± SEM. * $p<0.05$. The resting platelet $[Ca^{2+}]_i$ was significantly higher in diabetes than that in nondiabetics.

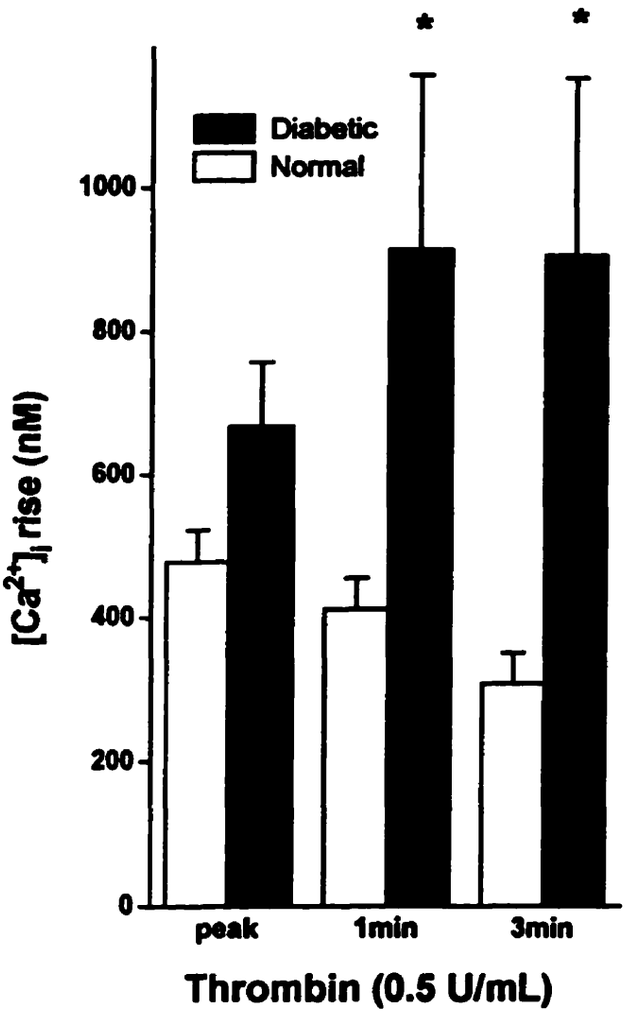


Figure 5

Thrombin-induced platelet $[Ca^{2+}]_i$ response in the presence of 1 mM extracellular Ca^{2+}. Nondiabetic group(n=9, open column) and Diabetic group(n=9, black column). Platelets were loaded with fura-2 AM. Values are means $\pm$ SEM. * $p<0.05$. Thrombin (0.5U/mL) induced increase in platelet $[Ca^{2+}]_i$ at 3 different time points: peak 1, 1 minute and 3 minutes $[Ca^{2+}]_i$ after the peak. There were no significant difference between peak 1 $[Ca^{2+}]_i$ response between nondiabetic group and diabetic group. The platelet at 1 and 3 minutes after the peak was higher in diabetes compared to nondiabetes.

2 μg/ml to 20μg/ml) in both diabetes and nondiabetes. For each dose the $[Ca^{2+}]_i$ rise was significantly higher in the platelets from diabetic group than that of the nondiabetic group.

In the presence of external calcium, the source of agonist stimulated $[Ca^{2+}]_i$ increases could be from intracellular Ca^{2+} store release and/or Ca^{2+} influx from the extracellular medium. To investigate the mechanisms responsible for the enhanced agonist-induced $[Ca^{2+}]_i$ response in the diabetic state, we tested whether the platelet intracellular Ca^{2+} store and release are affected by diabetes.

INTRACELLULAR STORE SIZE AND AGONIST-RELEASABLE CA^{2+}

As described in the methods, the intracellular releasable Ca^{2+} store size can be estimated by the peak $[Ca^{2+}]_i$ increase induced by the maximal dose of ionomycin in the absence of extracellular Ca^{2+}. Figure 7 shows that the peak $[Ca^{2+}]_i$ response to 5μM ionomycin in Ca^{2+}-free medium did not differ between platelets from normals and diabetics. This suggests that in platelets from diabetics, the total amount of releasable Ca^{2+} in the intracellular calcium stores (DTS) was not different from that of nondiabetic subjects.

When thrombin (0.5U/mL) was applied in Ca^{2+}-free medium, the peak intracellular Ca^{2+} release was also not significantly different between platelets from diabetic patients and nondiabetic subjects (figure 8). This indicates that thrombin releasable Ca^{2+} is also not changed in diabetes. In this condition, the $[Ca^{2+}]_i$ at 1 minute after the peak was higher in

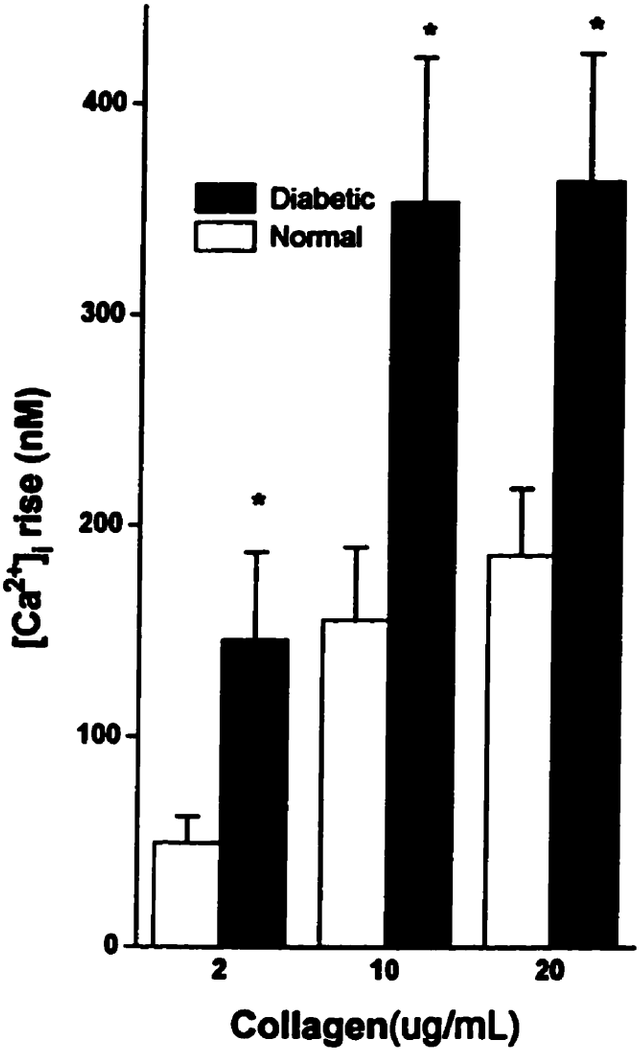


Figure 6

Collagen induced platelet $[Ca^{2+}]_i$ rise at 3 different doses (2 , 10, and 20 μg/ml) in nondiabetic subjects(n=9, open column) and diabetic patients (n=9, black column). The collagen-induced $[Ca^{2+}]_i$ rise was calculated by subtracting basal $[Ca^{2+}]_i$ from the maximal $[Ca^{2+}]_i$ after addition of collagen.

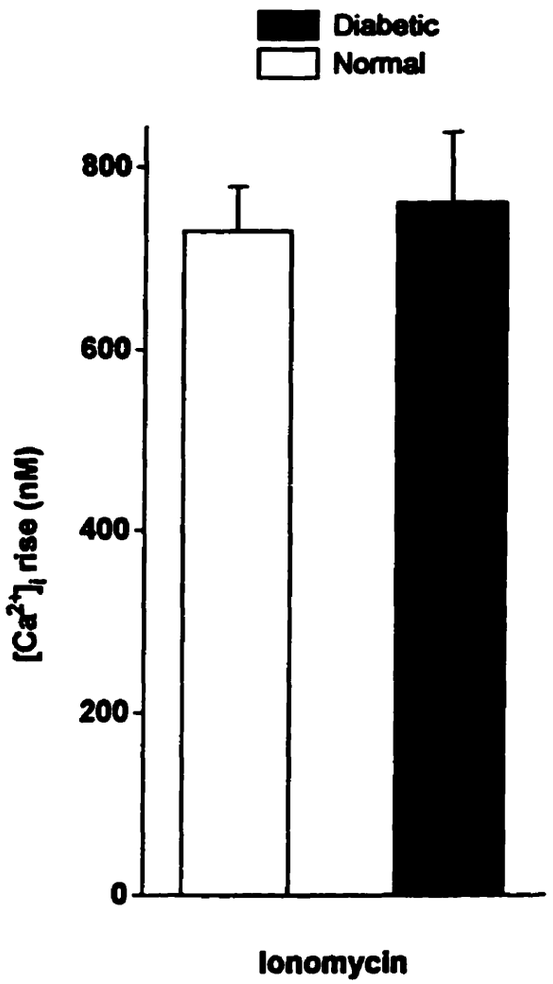


Figure 7

Ionomycin-stimulated $[Ca^{2+}]_i$ rise in the absence of external Ca^{2+} in fura-2 loaded platelets. Platelets were first suspended in 1 mM external Ca^{2+} for 3 minutes, then 5 mM EGTA was added to chelate the extracellular Ca^{2+}. After that a maximal dose (5 μM) of ionomycin was added and produced an immediate rise in $[Ca^{2+}]_i$. The peak of ionomycin-induced $[Ca^{2+}]_i$ rise did not differ between platelets from nondiabetic group (n=9, open column)and diabetic group(n=9, black column).

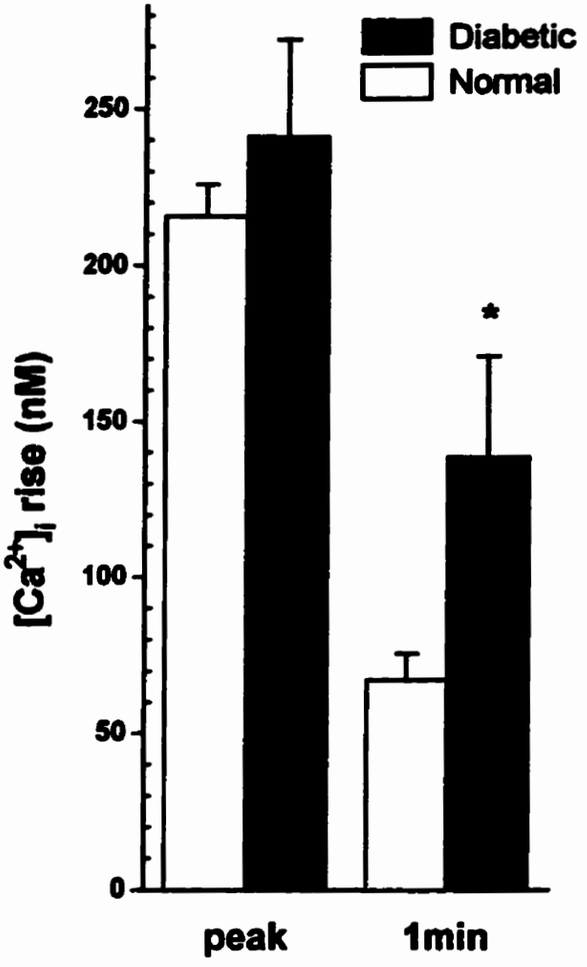


Figure 8

Thrombin-induced $[Ca^{2+}]_i$ rise in absence of external Ca^{2+}. Platelets were suspended in 1 mM external Ca^{2+} for 3 minutes then 5 mM EGTA was added to chelate the extracellular Ca^{2+}. Then 0.5U/ml thrombin was applied. The peak of thrombin-induced $[Ca^{2+}]_i$ rise was not different between nondiabetes and diabetes, the $[Ca^{2+}]_i$ level at 1 minute after the peak was significantly higher in the platelets from diabetic group(n=9) compared to that from normal group (n=9).

platelets from diabetics compared with controls suggesting that the rate of cytosolic $[Ca^{2+}]_i$ recovery after thrombin stimulation was slower in platelets from diabetes.

In the absence of extracellular Ca^{2+}, the recovery of the Ca^{2+} signal after thrombin stimulated Ca^{2+} release indicates removal of Ca^{2+} from the cytosol into internal stores or extrusion across the plasma membrane. If the rate of this $[Ca^{2+}]_i$ recovery process is slower, then it suggests that the extrusion and/or sequestration mechanisms are impaired. In platelets, there are two mechanisms for Ca^{2+} extrusion: plasma membrane Ca^{2+}-ATPase and Na^+-Ca^{2+} exchanger.

In type I diabetes Bergh (1988) showed that ^{45}Ca influx into saponized platelets was equal to that seen in controls, indicating that the uptake of Ca^{2+} into DTS was not different in the two groups. It has also been reported that in both type I and type II diabetic patients, platelet plasma membrane Ca^{2+}-ATPase activity was higher compared with control subjects (Mazzanti et al., 1990). These findings led us to consider the Na^+-Ca^{2+} exchanger as the possible candidate for the observed abnormal Ca^{2+} handling in platelets from diabetes. We next focused on the Na^+-Ca^{2+} exchanger to determine whether it is altered and involved in the enhanced agonist-stimulated platelet $[Ca^{2+}]_i$ response in diabetes. Before testing the pathological role of the platelet Na^+-Ca^{2+} exchanger in diabetes, it is first essential to confirm the existence of this exchanger in human platelets and to determine the physiological role.

PART II: NA^+-CA^{2+} EXCHANGER IN PLATELETS FROM NORMAL SUBJECTS AND DIABETIC PATIENTS

Na^+-Ca^{2+} Exchanger in Platelets from Normal Subjects

The physiological importance of the Na^+-Ca^{2+} exchanger in the regulation of $[Ca^{2+}]_i$ has been demonstrated in many cells such as cardiac muscle and photoreceptors. In the literature, there are several reports concerning the existence of the Na^+-Ca^{2+} exchanger in human platelets, but the findings have been variable and controversial. In our studies, we used several approaches to confirm the existence of the Na^+-Ca^{2+} exchanger and determined its role in regulation of platelet cytosolic calcium concentration.

Existence of Na^+-Ca^{2+} exchanger in human platelets

The existence of the Na^+-Ca^{2+} exchanger was confirmed by the effects of reducing the transmembrane Na^+ gradient on platelet Ca^{2+} homeostasis and these results are shown in Figure 9 and Table 2. Figure 9 shows a typical trace in which platelet basal $[Ca^{2+}]_i$ and thrombin-stimulated calcium responses were measured in 4 conditions. A. Control: 140mM $[Na^+]_o$ medium was used as control, in which platelets were suspended in standard buffer (140mM). B. Ouabain: Platelets were pretreated with 0.1 mM Ouabain to inhibit Na-K ATPase and increase intracellular Na^+ concentration; C. Low $[Na^+]_o$: 28mM external Na^+ medium, as described in the method the external Na^+ was partially replaced by isomolar NMDG, thereby reducing the Na^+ gradient by decreasing extracellular Na^+

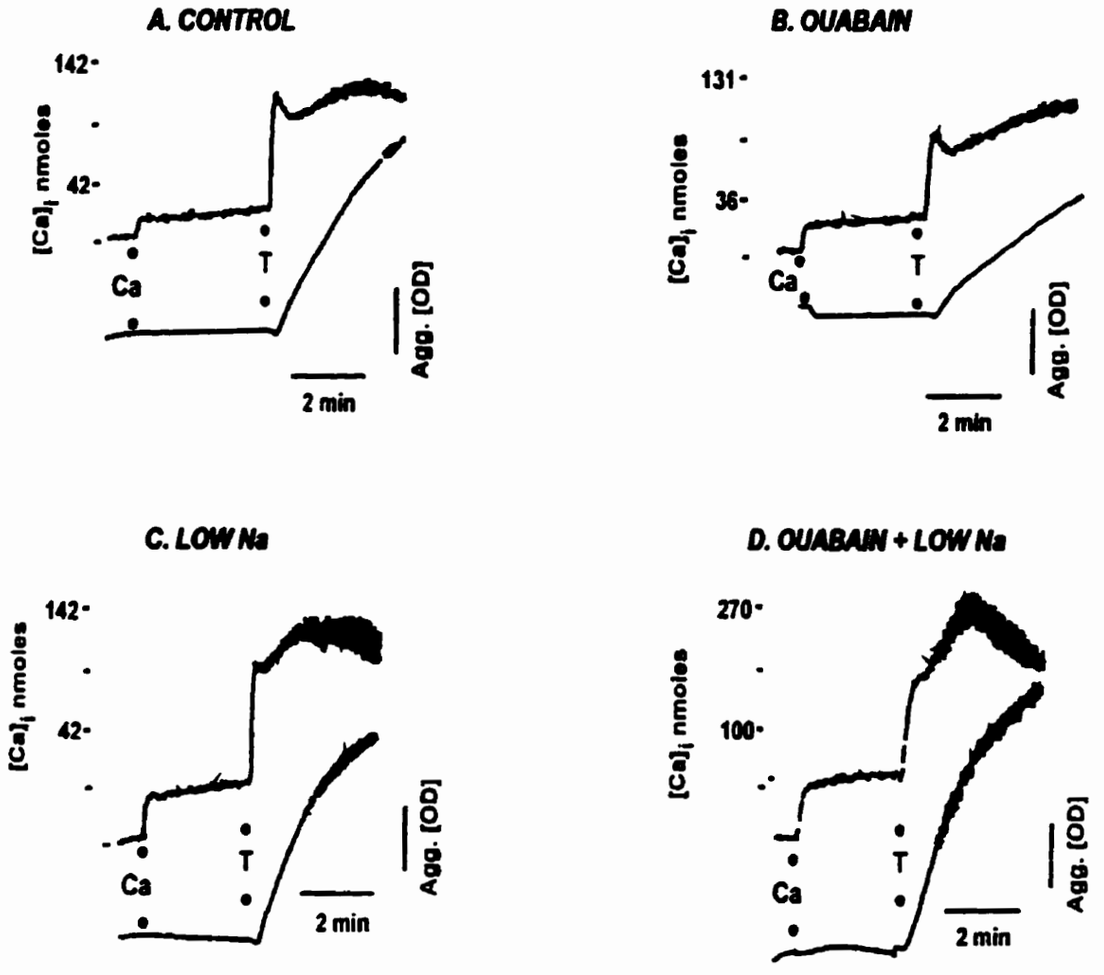


Figure 9

Representing trace showing the effects of reducing transmembrane Na^+ gradient on resting $[Ca^{2+}]_i$ and thrombin (0.5U/ml)-stimulated $[Ca^{2+}]_i$ rise and platelet aggregation in the presence of 1 mM external Ca^{2+}. A. Control, normal extracellular Na^+ medium (140mM). B. Pretreatment of platelets with 0.1 mM ouabain. C. Low (28 mM) external Na^+ medium. D. A combination of low Na^+ medium and pretreatment with ouabain. Low Na^+ medium enhanced platelet resting $[Ca^{2+}]_i$ and phase 2 of thrombin-induced $[Ca^{2+}]_i$ rise and aggregation. Ouabain treatment alone had no effects on $[Ca^{2+}]_i$. Low Na^+ and Ouabain together can amplify the effect of low Na^+ alone.

concentration. D. Ouabain + Low Na^+: A combination of 28 mM $[Na^+]_o$ and pretreatment of 0.1 mM/L Ouabain that should decrease Na^+ gradient more.

Table 2: Effect of reducing transmembrane Na^+ gradient on platelet basal $[Ca^{2+}]_i$

	Basal $[Ca^{2+}]_i$ (nM±SE(n))
A. Control (140 mM $[Na^+]_o$)	36.7±5.4 (15)
B. Low Na (28 mM $[Na^+]_o$)	55.4±10.7(15)
C. Ouabain pretreatment	43.4±6.53 (12)
D. B+C treatment	66.3±11.8 (12)

P values: A vs B: <0.05; A vs C: NS; B vs D: NS; A vs D: <0.05; C vs D: <0.05. Platelets were loaded with Ca-green/fura-red. Basal platelet $[Ca^{2+}]_i$ was determined once the $[Ca^{2+}]_i$ stabilized after 3 minutes following addition of 1 mM $CaCl_2$ as described in the methods.

Table 2 summarizes the basal $[Ca^{2+}]_i$ values under the above mentioned 4 conditions. In 28 mM external Na^+ medium, basal $[Ca^{2+}]_i$ level increased significantly compared with the normal (140 mM) external medium. There was no significant difference in basal $[Ca^{2+}]_i$ between control and ouabain pretreatment. However in the condition of low $[Na^+]_o$ with ouabain pretreatment, basal $[Ca^{2+}]_i$ levels was higher than low $[Na^+]_o$ alone.

Table 3: Effect of reducing transmembrane Na^+ gradient on thrombin-induced $[Ca^{2+}]_i$ response

	Peak 1 (nM±SE(n))	Phase 2 (nM±SE(n))
A. Control (140mM $[Na^+]_o$)	283.61±33.15(6)	269.78±55.16(7)
B. Low Na (28mM $[Na^+]_o$)	327.36±35.37(6)	369.67±66.02(7)
C. Ouabain pretreatment	299.39±24.47(6)	288.15±59.02(7)
D. B+C treatment	345.62±34.50(6)	407.65±69.49(7)

P values: Peak 1: A vs B:NS; A vs C:NS; B vs D: NS; C vs D:NS. Phase 2: A vs B:<0.01; A vs C:NS; B vs D:NS; C vs D:<0.01. Thrombin (0.5U/mL) stimulated peak1 and phase 2 $[Ca^{2+}]_i$ responses were determined as shown in Figure 2.

The thrombin-stimulated $[Ca^{2+}]_i$ responses under these 4 conditions are given in Table 3. Under all these conditions, there was no significant difference in the peak 1 of $[Ca^{2+}]_i$ response to thrombin (0.5U/ml) stimulation. The phase 2 ($[Ca^{2+}]_i$ at 1 minute after the peak) increased significantly in the presence of low Na^+. Similar to basal $[Ca^{2+}]_i$, pretreatment of platelets with ouabain had no effect on thrombin-induced cytosolic $[Ca^{2+}]_i$ rise. If low Na^+ was combined with pretreatment with ouabain, the phase 2 of the thrombin response increased dramatically, the $[Ca^{2+}]_i$ being larger than lowering extracellular Na^+ alone.

In the low Na^+ medium, because the extracellular Na^+ was replaced by NMDG, the results could be interpreted in 2 ways. One is that the effect could be due to a decrease in extracellular Na^+ concentration. Another explanation could be that the effect of this medium was due to the specific action of NMDG. To investigate the latter possibility, we used another agent, sucrose, to replace extracellular Na^+ and compared the effects of these 2 media in the same batch of loaded platelets. The results from 4 batches of platelets from 4 individuals are shown in Table 4. In either NMDG or sucrose media, thrombin-induced peak 1 calcium response was not significantly different from control. As far as phase 2 is concerned, $[Ca^{2+}]_i$ at 1 minute after the peak was 172.23$\pm$ 29.87 (n=4) in 140 mM Na^+. Substitution of external Na^+ with NMDG increased this $[Ca^{2+}]_i$ to 270.74$\pm$52.71 nM (n=4). If external Na^+ was replaced by sucrose, then the $[Ca^{2+}]_i$ was 279.23$\pm$26.67nM (n=4). Compared to the control, the increase of the phase 2 of thrombin-induced $[Ca^{2+}]_i$ response in NMDG or sucrose media are significant. There was no difference between NMDG and sucrose. These results thus excluded the possibility that the effect of low Na^+ was due to the effect of the specific agent used for replacement. Thus, the increases in $[Ca^{2+}]_i$ appear to be due to a decrease in Na^+ gradient across the plasma membrane and this can lead to a decrease in the activity of the Na^+-Ca^{2+} exchanger. In summary, these results showed that lowering extracellular Na^+ can increase basal $[Ca^{2+}]_i$ and thrombin-induced phase 2 $[Ca^{2+}]_i$ response by decreasing the Na^+ gradient.

Table 4: Comparison of the effect of NMDG and sucrose substituted low Na^+ media on thrombin-induced $[Ca^{2+}]_i$ response

	Phase 2 (nM±SE(n))
A. Control (140mM $[Na^+]_o$)	172.23±29.87(4)
B_1. 28mM $[Na^+]_o$ (Sucrose)	279.23±26.67(4)
B_2. 28mM $[Na^+]_o$ (NMDG)	270.74±52.71(4)
C. Ouabain treatment	183.15±28.27(4)
D_1. B_1 + C	308.37±56.79(4)
D_2. B_2 + C	283.38±53.13(4)

P values: A vs B_1: <0.05; A vs B_2: <0.05; B_1 vs B_2: NS; D_1 vs D_2: NS; C vs D_1:<0.05; C vs D_2: <0.05.

To determine whether altering the Na^+ gradient would affect mobilization of $[Ca^{2+}]_i$ from intracellular stores or Ca^{2+} flux across the plasma membrane, experiments were carried out in Ca^{2+}-free medium (EGTA was added to chelate extracellular Ca^{2+}) in the above 4 conditions. As shown in Figure 10, in the absence of extracellular Ca^{2+}, thrombin (0.5U/ml) addition produced a rapid transient rise of $[Ca^{2+}]_i$ which then returned to basal levels. The peak of this $[Ca^{2+}]_i$ rise reflects only Ca^{2+} release from intracellular

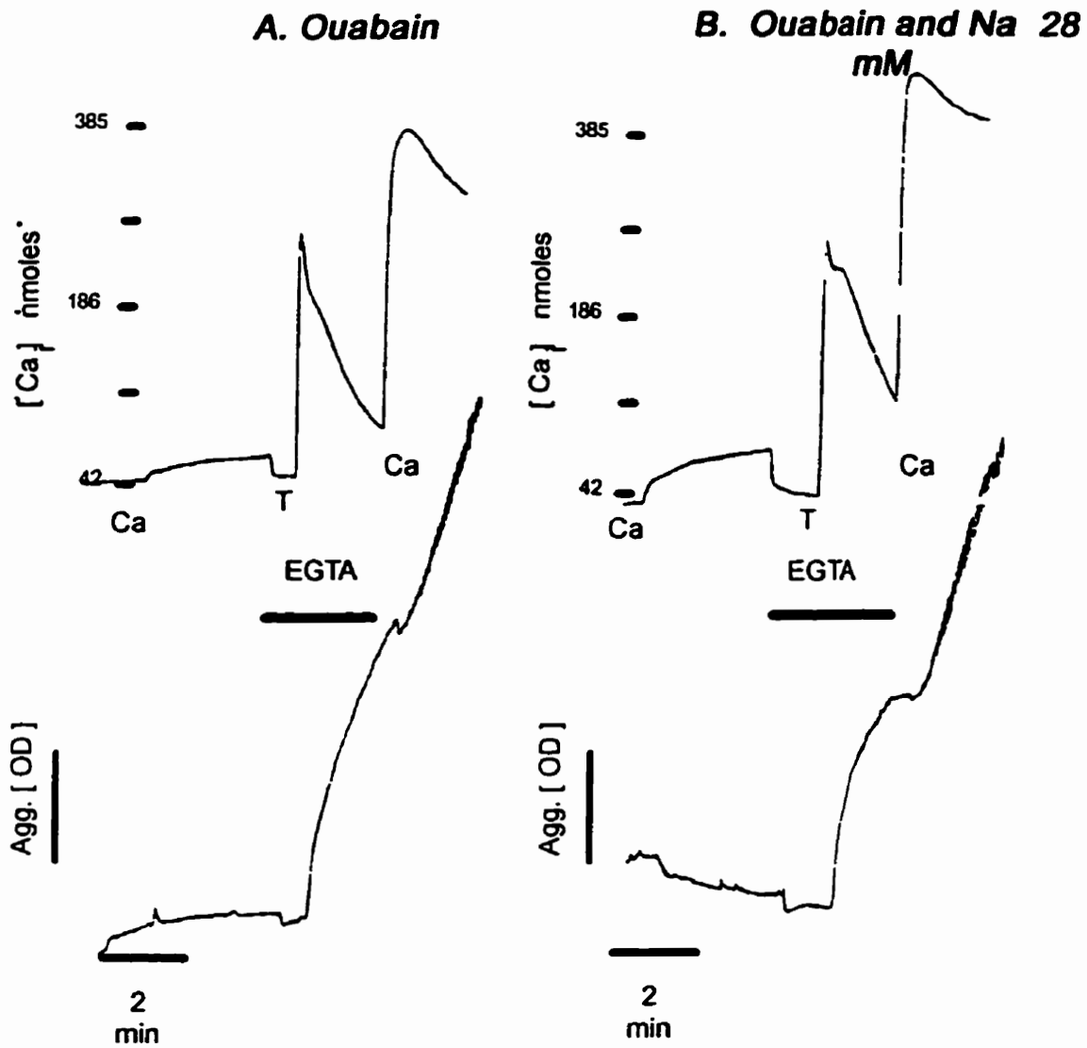


Figure 10

Representing trace showing the effect of reducing Na^+ gradient on thrombin-induced $[Ca^{2+}]_i$ increase and aggregation in the absence of external Ca^{2+}. After adding 1 mM $CaCl_2$ for 3 minutes, 5 mM EGTA was added to remove external Ca^{2+}, then 0.5U/ml thrombin was added showing thrombin-induced intracellular Ca^{2+} release. After thrombin response, 10mM $CaCl_2$ was added showing Ca^{2+} influx. A. normal Na^+ and Ouabain B. low Na^+ (28mM) medium and Ouabain. The peak of thrombin-induced Ca^{2+} release was not affected by low Na^+ medium while the $[Ca^{2+}]_i$ at 1 minute after the peak was higher in low Na^+ medium.

stores. The $[Ca^{2+}]_i$ decline after the peak indicates the removal process of cytosolic Ca^{2+} after thrombin stimulation. Table 5 summarizes the data. There was no significant difference in the thrombin-induced Ca^{2+} release among these 4 conditions. The $[Ca^{2+}]_i$ level at 1 minute after the peak was increased under low Na^+ (190.93±34.23mM, n=5) compared to the control (129.24±20.78, n=5). Similar to the experiments in the presence of extracellular Ca^{2+}, ouabain pretreatment had no effect on $[Ca^{2+}]_i$. Ouabain plus low Na^+ amplified this increase (from 129.24±20.78nM in control to 271.79±46.65nM, n=5). These data suggest that reducing the Na^+ gradient does not affect Ca^{2+} release from intracellular store, but affects the cytosolic Ca^{2+} recovery process after the release. As noted above, this recovery of cytosolic Ca^{2+} might reflect Ca^{2+} extrusion or/and sequestration mechanisms.

Table 5: Effect of reducing transmembrane Na^+ gradient on thrombin-induced Ca^{2+} response in absence of extracellular Ca^{2+}

	Peak 1 (nM±SE(n))	Phase 2 (nM±SE(n))
A. Control (140mM $[Na^+]_o$)	217.54±41.52(5)	129.24±20.78(5)
B. Low (28 mM $[Na^+]_o$)	216.64±40.37(5)	190.93±34.23(5)
C. Ouabain pretreatment	249.94±63.38 (5)	156.99±40.34(5)
D. B + C treatment	242.02±63.15(5)	271.79±46.65(5)

P values: Peak1: all comparisons:NS; Phase 2: A vs B:<0.05; A vs C: NS; C vs D: <0.05.

These results showed that reducing the Na^+ gradient across the plasma membrane can produce an increase in platelet cytosolic $[Ca^{2+}]_i$ concentration in both resting and thrombin-stimulated conditions. confirming that a Na^+-Ca^{2+} exchanger mechanism exists in the platelet plasma membrane. After the Na^+ gradient is decreased, cytosolic $[Ca^{2+}]_i$ increased, suggesting that the role of the Na^+-Ca^{2+} exchanger is to mediate Ca^{2+} efflux in the resting and thrombin-stimulated conditions. We also found that after reducing the Na^+ gradient, intracellular Ca^{2+} release is not changed but the recovery process is slower, similar to the platelet calcium abnormality in diabetes that we observed in the first part of this thesis. This result implies that in platelets from diabetics the Na^+-Ca^{2+} exchanger may be impaired. We next used a specific inhibitor of the Na^+-Ca^{2+} exchanger to study its physiological and pathological roles.

The physiological role of Na^+-Ca^{2+} exchanger in platelet calcium homeostasis

In order to establish the physiological role of the Na^+-Ca^{2+} exchanger in platelet Ca^{2+} homeostasis, a specific inhibitor had to be used. Among amiloride analogues, CBDMB is the most potent and the most specific(Zhuang et al., 1984; Lazdunski, et al., 1985; Simchowitz and Cragoe, 1986; Kaczorowski, et al., 1985). The effects of CBDMB in platelets were tested on the Na^+-concentration-dependent $[Ca^{2+}]_i$ decline that represented the forward mode of the Na^+-Ca^{2+} exchanger. As shown in Figure 11, The gel-filtered platelets were resuspended in 0 Na^+ medium (completely replaced by NMDG)

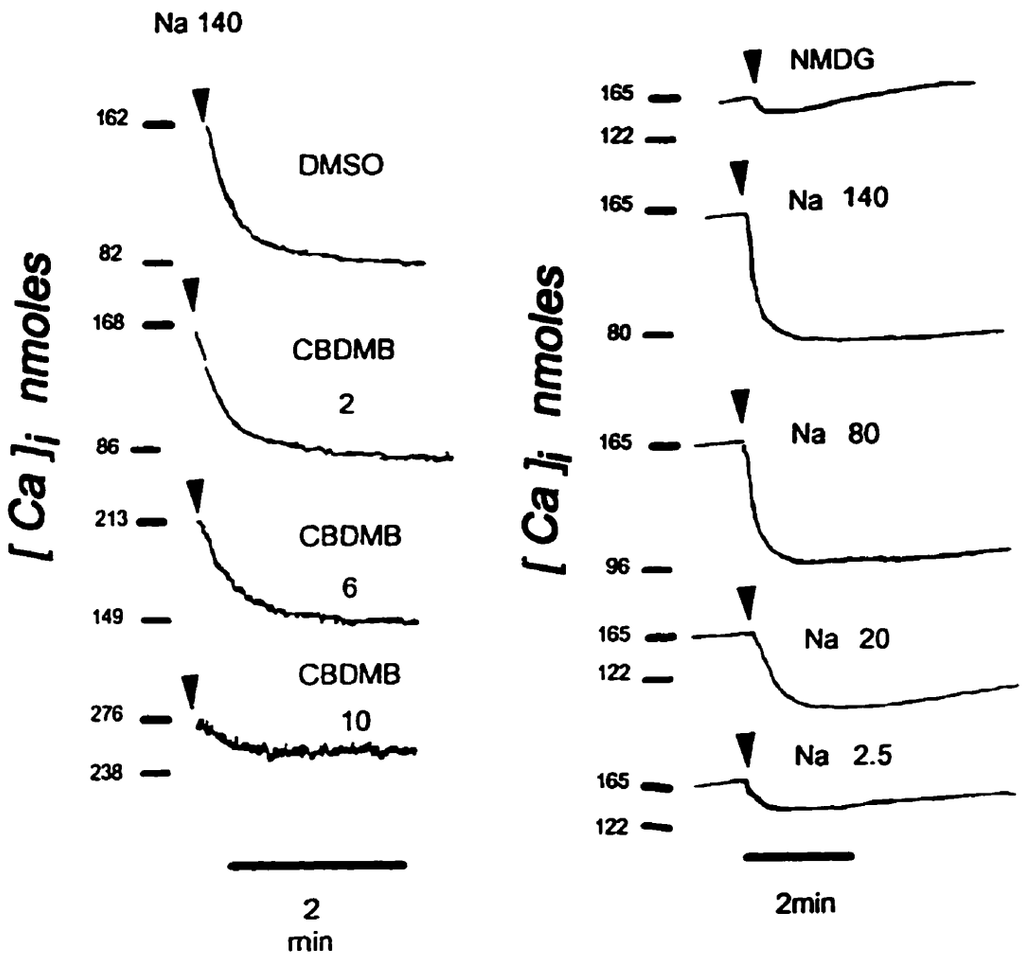


Figure 11

Effect of replenishment of external Na^+ on $[Ca^{2+}]_i$ of platelets suspended in zero-Na^+ and 1 mM Ca^{2+} containing medium. Platelets were equilibrated with 1 mM Ca^{2+} for 15 minutes prior to addition of external Na^+. Right trace: Different concentrations of $[Na^+]_o$ induced $[Ca^{2+}]_i$ decline. N-methyl D-glucamine was used to balance the ionic strength of $[Na^+]_o$. $[Ca^{2+}]_i$ was 165 nM in all right traces. Left trace: Effect of different concentrations of CBDMB on 140 mM $[Na^+]_o$ induced $[Ca^{2+}]_i$ decline.

and then equilibrated with 1 mM external Ca^{2+}. When the $[Ca^{2+}]_i$ reached a plateau level, different concentrations of NaCl were added extracellularly and there was a Na^+-dependent $[Ca^{2+}]_i$ decline. The right trace of Figure 11 shows after $[Ca^{2+}]_i$ reached a plateau level (about 165nM), NaCl was applied to the medium to final concentrations from 140 mM to 2.5 mM to induce a $[Na^+]_o$ dose-dependent $[Ca^{2+}]_i$ decline. Addition of 140 mM NMDG had no effect on $[Ca^{2+}]_i$ indicating the effect of external Na^+ is not due to osmolarity change. In intact platelets if $[Ca^{2+}]_i$ decreases, it may reflect Ca^{2+} reuptake into the DTS or/ and Ca^{2+} efflux across the plasma membrane. Because this $[Ca^{2+}]_i$ decline is Na^+-dependent, and cyclopiazonic acid (CPA) which is an inhibitor of Ca-ATPase in DTS, did not abolish this decline (data not shown), this external Na^+ dependent Ca^{2+} efflux can be used to represent the forward mode of Na^+-Ca^{2+} exchanger. From this mode we calculated the apparent K_m of the exchanger for external Na^+ as 27 mM$\pm$ 3.00 (n=5). Next we used this mode to test the specificity of CBDMB.

The left trace of figure 11 shows that CBDMB inhibited this Na^+-dependent $[Ca^{2+}]_i$ decline in a dose dependent manner (from 2μM to 10 μM). In the vehicle control (DMSO), 140 mM external Na^+ caused a 80 nM $[Ca^{2+}]_i$ decline (from 162 nM to 82 nM). If platelets were pretreated with 10 μM CBDMB, the same dose of external Na^+ only produced a 20 nM $[Ca^{2+}]_i$ decrease. This result suggests that in intact platelets CBDMB can inhibit Na^+-Ca^{2+} exchanger in the μM range, which is similar to the reported K_i value of 7.3 μM of CBDMB in other tissues.

In addition to Na^+-Ca^{2+} exchanger there are other Na^+-dependent ion transporters in intact platelets such as the Na^+-H^+ exchanger. The possibility that CBDMB could inhibit the Na^+-H^+ exchanger should be assessed. The effect of CBDMB on the platelet Na^+-H^+ exchanger was compared with a known specific Na^+-H^+ exchanger inhibitor, EIPA (Zhuang et al., 1985; Lazdunski et al., 1985). As shown in Figure 12, the platelets were loaded with a pH-sensitive dye (BCECF) and the cytosolic pH was measured. First platelets were resuspended in 0 Na^+ medium and then 140 mM external NaCl was added. This caused a Na^+-dependent $[pH]_i$ rise (alkalization) which represents the Na^+-H^+ exchanger. EIPA (10μM) inhibited the Na^+-H^+ exchanger. CBDMB (1 and 10μM) had no effect on Na^+-H^+ exchanger activity. These results indicated that CBDMB, at concentrations that inhibit the Na^+-Ca^{2+} exchanger, had no inhibiting effect on the platelet Na^+-H^+ exchanger. Therefore, CBDMB is a specific inhibitor of the Na^+-Ca^{2+} exchanger in platelets and it can be used to study the physiological and pathological role of the Na^+-Ca^{2+} exchanger.

In order to determine the physiological role of Na^+-Ca^{2+} exchanger in platelet Ca^{2+} homeostasis, the effects of CBDMB on platelet resting and thrombin-induced $[Ca^{2+}]_i$ responses were studied. Figure 13 shows the dose-response curve of CBDMB at increasing resting $[Ca^{2+}]_i$ in the presence of normal external Na^+ and in zero Na^+ medium. As CBDMB quenches fura-2, calcium-green/fura-red were used to measure $[Ca^{2+}]_i$ in the platelets in all the experiments in which CBDMB was used. The absolute $[Ca^{2+}]_i$ values were lower than those in fura-2 loaded platelets, but the relative changes of $[Ca^{2+}]_i$ were similar in fura-2 and Ca-green/fura-red loaded conditions. As shown here, in normal Na^+

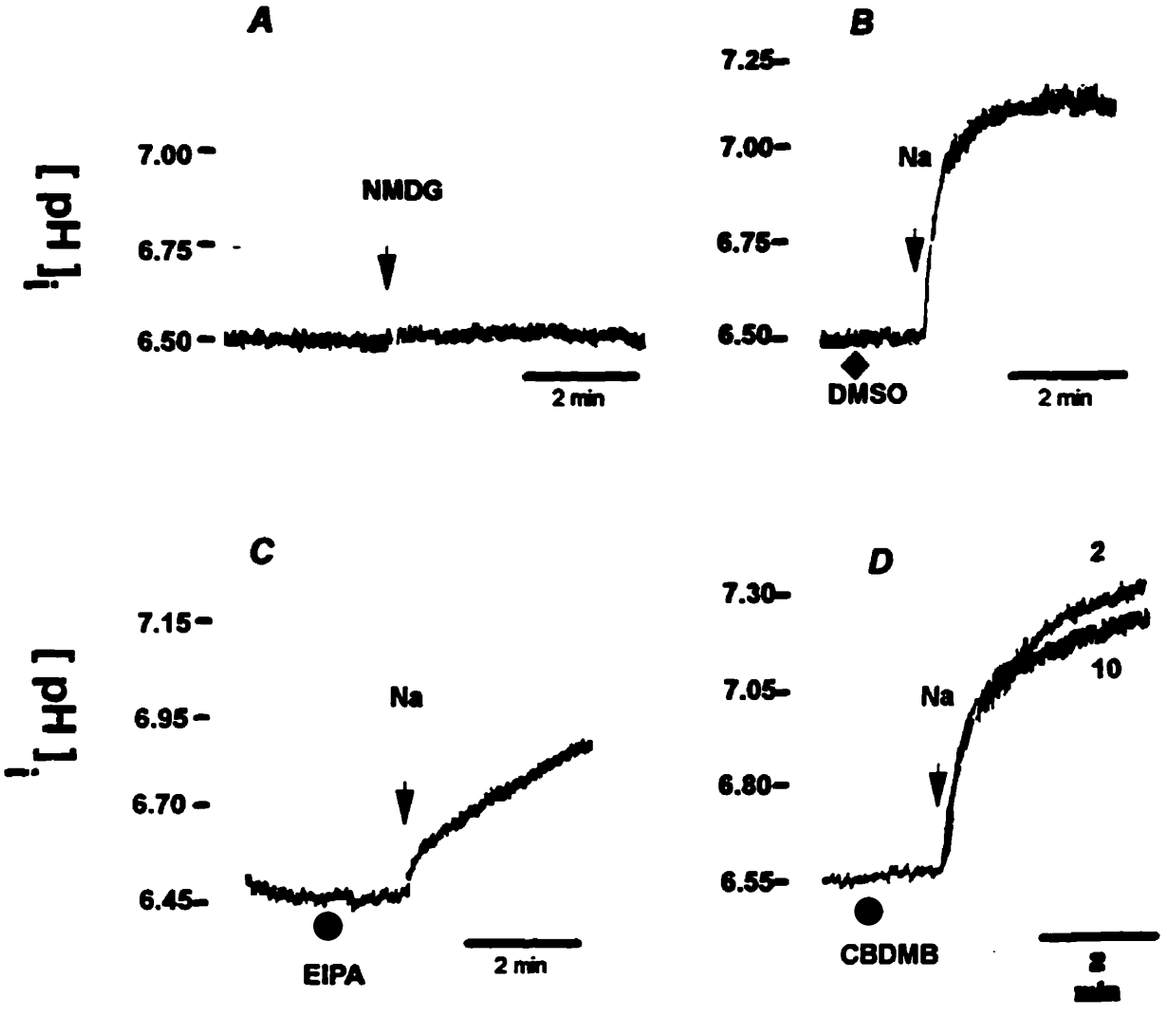


Figure 12

Effect of replenishment of $[Na^+]_o$ on $[pH]_i$ of platelets loaded with pH sensitive dye BCECF. A. Effect of adding 140 mM N-Methyl D-glucamine. B. Effect of adding 140 mM $[Na^+]_o$. C. Effect of 10 μM EIPA on Na^+-induced increase in $[pH]_i$. D. Effect of 2 μM and 10 μM CBDMB, showing no effect on intracellular pH recovery.

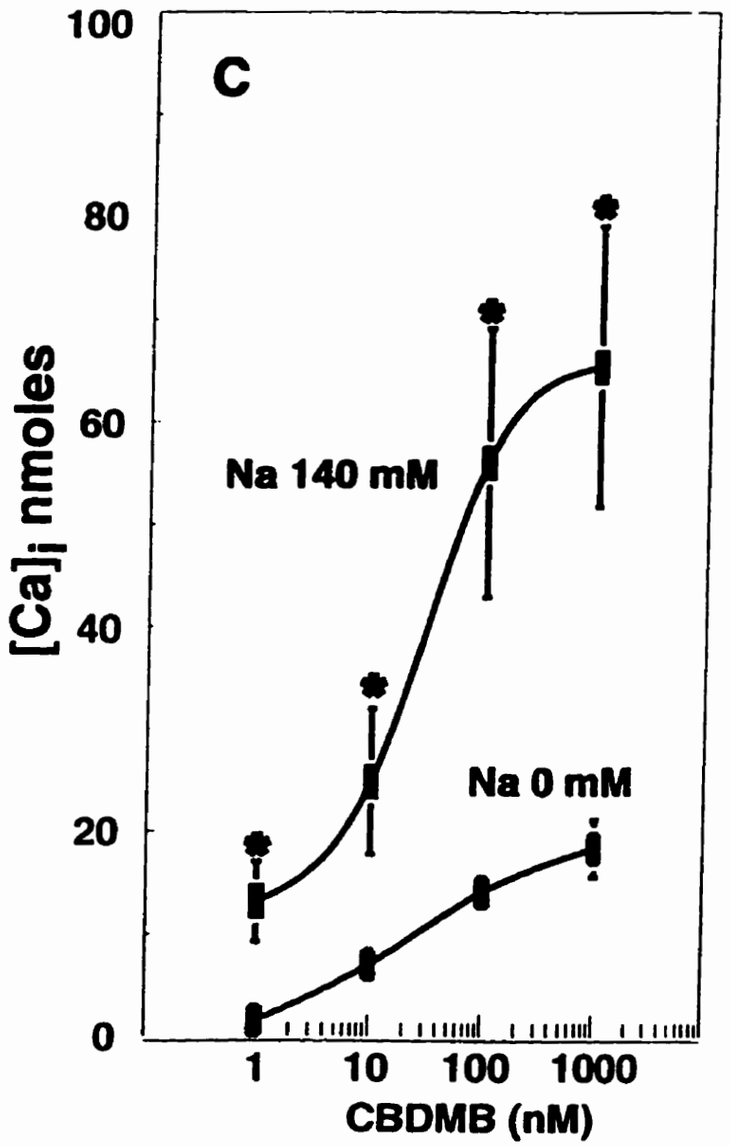


Figure 13

Effect of CBDMB on platelet resting $[Ca^{2+}]_i$ and its dependence on external Na^+. In presence of 140 mM external Na^+ medium, CBDMB increased resting $[Ca^{2+}]_i$ in a dose dependent manner. In the absence of external Na^+ (NaCl was replaced by isomolar NMDG), CBDMB had no effect on resting $[Ca^{2+}]_i$. Comparing each dose of CBDMB in presence of 140 mM Na^+ and zero Na^+ medium, the difference is significant ($P<0.05$).

medium, basal $[Ca^{2+}]_i$ increased after CBDMB pretreatment. The increase is dependent on the dose of CBDMB. If the external medium was depleted of Na^+ (completely substituted by NMDG), CBDMB had no effects on resting $[Ca^{2+}]_i$. The data comparing each dose of CBDMB in the presence of normal Na^+ and zero Na^+ media were significantly different. Therefore this result suggests that in the resting state the role of the platelet Na^+-Ca^{2+} exchanger is to mediate Ca^{2+} efflux (working in forward mode). because as described in the method (Figure 3), if the exchanger mediates Ca^{2+} efflux, after inhibition by CBDMB, cytosolic $[Ca^{2+}]_i$ should increase. When external Na^+ is zero the internal Na^+ will be depleted and the Na^+-Ca^{2+} exchanger cannot function, so in this condition CBDMB had no effect on basal $[Ca^{2+}]_i$.

Figure 14 shows the effect of CBDMB on the response of $[Ca^{2+}]_i$ following stimulation with thrombin. DMSO, the drug vehicle, was used as a control. The effect of CBDMB is similar to that of reducing transmembrane Na^+ gradient: the peak 1 of thrombin-induced $[Ca^{2+}]_i$ response was unaffected by CBDMB, but the phase 2 increased significantly after the platelets were pretreated with 2 μM CBDMB. These data indicate that in normal platelets, under the stimulation of thrombin, the function of the Na^+-Ca^{2+} exchanger is to remove Ca^{2+} out of the cell (forward mode of Na^+-Ca^{2+} exchanger), because when the Na^+-Ca^{2+} exchanger activity was inhibited by CBDMB, cytosolic $[Ca^{2+}]_i$ increased.

In addition, we used another CBDMB blocker, XIP (eXchanger Inhibitory Peptide), to confirm the results seen with CBDMB. XIP was synthesized and

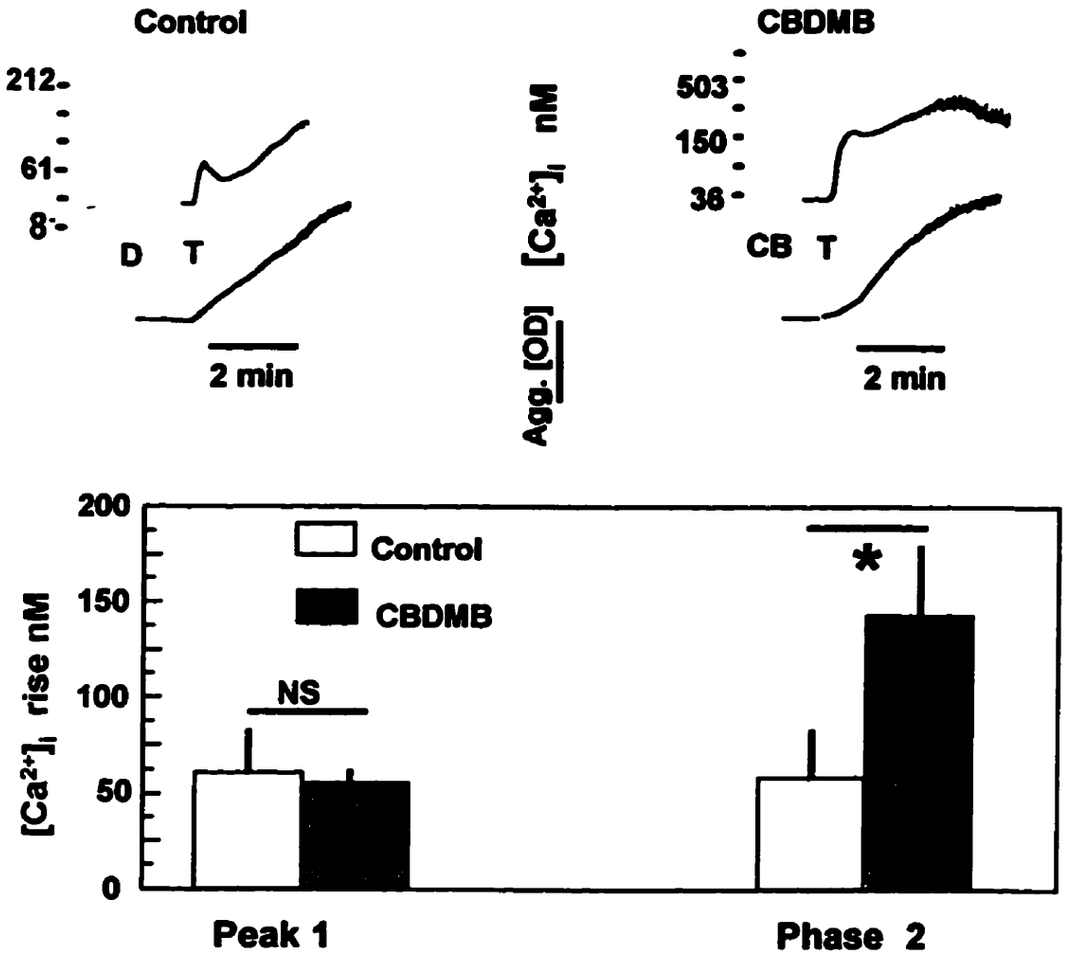


Figure 14

Potentiating effect of CBDMB on thrombin-induced $[Ca^{2+}]_i$ rise and aggregation in platelets from normal subjects. Top: a typical trace showing CBDMB potentiated thrombin-stimulated $[Ca^{2+}]_i$ increase (the second phase) and aggregation. Platelets were treated with 1 μL DMSO (D as indicated) or 1 μL of 1 mM CBDMB (as indicated by CB) and 3 minutes later thrombin was added. A. DMSO control. B. 2 μM CBDMB. Bottom: summarized data from 5 experiments. There was no significant difference in peak 1 $[Ca^{2+}]_i$ rise between control (open bar)and after 2 μM CBDMB treatment(hatched bar). CBDMB increased the thrombin-induced phase 2 $[Ca^{2+}]_i$ rise significantly. Platelets were loaded with calcium-green and fura-red.

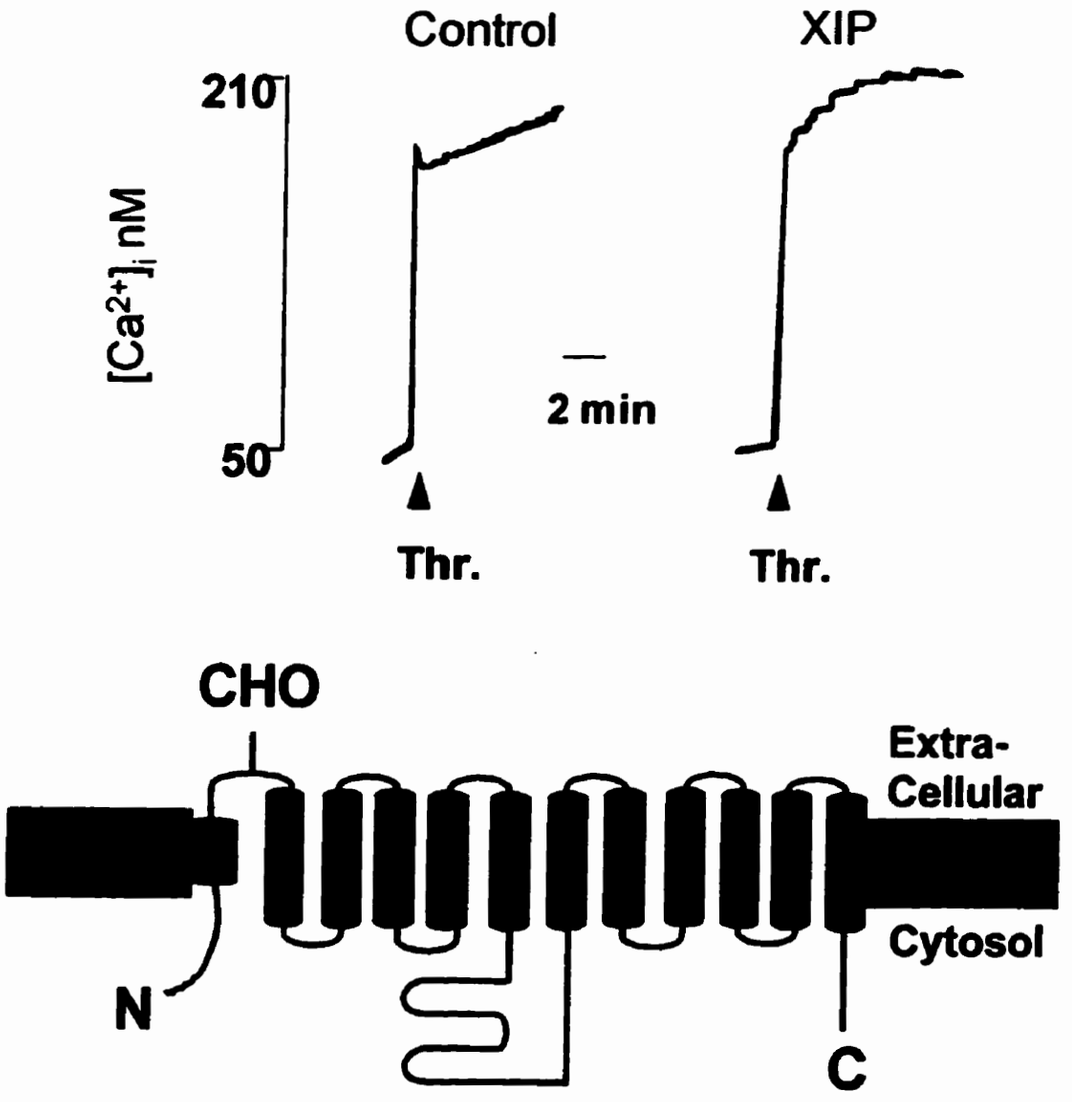


Figure 15

Potentiating effect of XIP on thrombin-induced $[Ca^{2+}]_i$ rise in platelets from normal subjects. XIP was loaded into the platelets as described in the text. In control the platelets went through the same procedure but loaded with the same volume of buffer. The bottom model shows the intracellular site of XIP on Na^{2+}-Ca^{2+} exchanger.

characterized by Dr. K.D. Philipson at UCLA. XIP must be inside the cell in order to inhibit the Na^+-Ca^{2+} exchanger. We used the same technique employed in our laboratory for loading aequorin in T-cells (Kraut et al., 1993) to load XIP. These cells are reversibly permeablized with EDTA and then exposed to XIP at 4° C for 30 min. Figure 15 shows that XIP increased thrombin-induced $[Ca^{2+}]_i$ rise, confirming that the Na^+-Ca^{2+} exchanger mediates Ca^{2+} efflux (forward mode) after calcium is increased by thrombin stimulation. In the control for the XIP loading experiment the platelets went through the same procedure of loading with the same volume of buffer as with XIP experiments.

All the results from 3 different approaches (low Na^+, CBDMB and XIP) suggest that the physiological role of the platelet Na^+-Ca^{2+} exchanger is to mediate Ca^{2+} efflux (forward mode) in the resting state and after thrombin stimulation. In the resting state, the role of the Na^+-Ca^{2+} exchanger is to maintain the resting platelet $[Ca^{2+}]_i$ to low levels. Following thrombin stimulation, the function of the Na^+-Ca^{2+} exchanger helps to remove cytosolic Ca^{2+} from the platelets.

Na^+-Ca^{2+} Exchanger in Platelets from Diabetic Patients

The above experiments have shown that CBDMB can be used to study the physiological role of the Na^+-Ca^{2+} exchanger in $[Ca^{2+}]_i$ homeostasis in intact platelets. Next we used CBDMB as a tool to determine whether the platelet Na^+-Ca^{2+} exchanger is different in diabetic patients and whether it is involved in the enhanced basal and agonist-(thrombin and collagen) stimulated $[Ca^{2+}]_i$ response observed in the first part of this thesis.

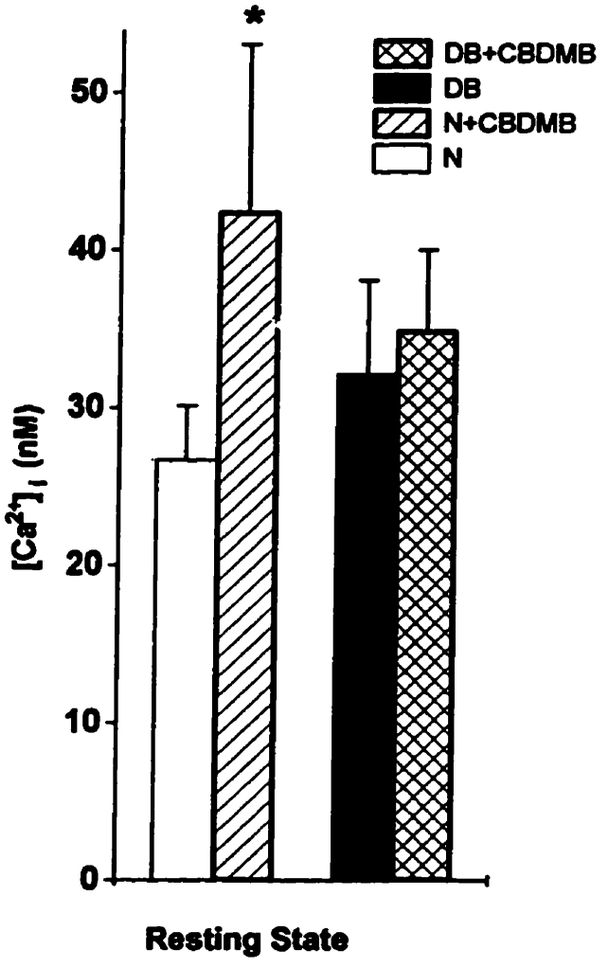


Figure 16

Effects of CBDMB on resting $[Ca^{2+}]_i$ response in platelets from nondiabetic group (N) and diabetic patients (DB). In platelets from nondiabetic group (n=9), CBDMB (2μM) increased basal platelet $[Ca^{2+}]_i$ significantly indicating that the Na^+-Ca^{2+}-exchanger works in the forward mode in the normal basal state. In platelets from diabetic patients (n=11), the same concentration of CBDMB had no effect on the basal $[Ca^{2+}]_i$ suggesting that the activity of the forward mode of Na^+-Ca^{2+}-exchanger was decreased.

First, the effect of CBDMB on basal $[Ca^{2+}]_i$ was studied. As shown in Figure 16, in platelets from nondiabetic subjects, addition of 2 μM CBDMB increased basal $[Ca^{2+}]_i$ significantly suggesting that the Na^+-Ca^{2+} exchanger works in the forward mode under resting conditions. In platelets from diabetic patients, addition of the same concentration of CBDMB produced no change in platelet basal $[Ca^{2+}]_i$, indicating the activity of the forward mode of the platelet Na^+-Ca^{2+} exchanger was decreased.

Secondly, the effect of CBDMB on thrombin-induced cytosolic $[Ca^{2+}]_i$ rise was tested. Figure 17 shows the effect of 2μM CBDMB on platelet $[Ca^{2+}]_i$ in diabetic patients. In contrast to the data in platelets from normal subjects in which 2μM CBDMB enhanced phase 2 of thrombin-induced $[Ca^{2+}]_i$ rise (Figure 14), in diabetes both peak 1 and phase 2 of thrombin-induced $[Ca^{2+}]_i$ rise were inhibited by CBDMB. This suggests that in platelets from diabetic patients, the Na^+-Ca^{2+} exchanger mediates Ca^{2+} influx (reverse mode), contributing at least in part to the enhanced thrombin-induced $[Ca^{2+}]_i$ response.

When the effect of CBDMB was expressed as a percent change of $[Ca^{2+}]_i$ of the control, a different role for the Na^+-Ca^{2+} exchanger in platelets from normal and diabetic subjects can be clearly seen in Figure 18. In nondiabetics the relative change after CBDMB was positive (138%,n=7), whereas in diabetics the relative change was negative (63%, n=14). This figure indicates that under thrombin-stimulated conditions the direction of the Na^+-Ca^{2+} exchanger is different in platelets from normal subjects and in those from diabetic patients.

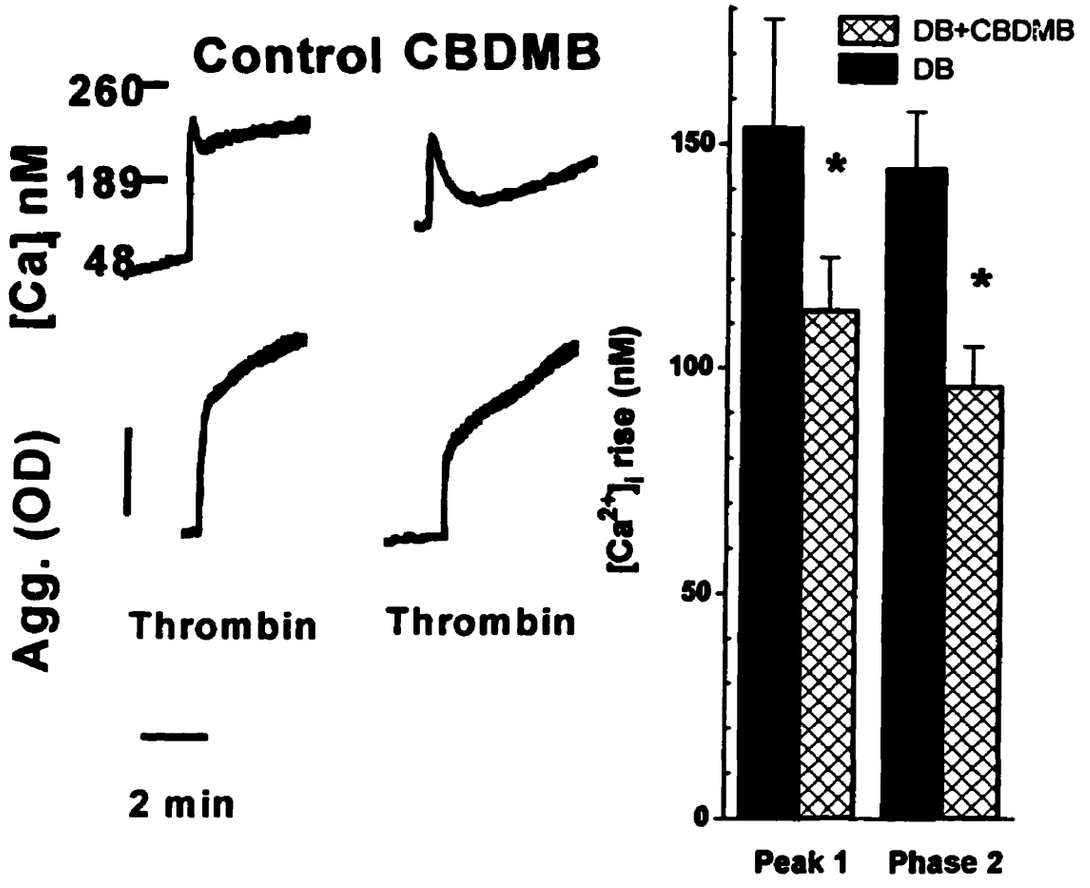


Figure 17

Inhibitory effects of CBDMB on thrombin-induced $[Ca^{2+}]_i$ rise in platelets from diabetic patients. Left: a typical trace from an uncontrolled type II patient. Platelets were isolated and loaded with the same conditions as for platelets from normal subjects. Also the treatment and concentration of CBDMB was the same as figure 14. Right: summarized data from 14 diabetic patients. CBDMB decreased both thrombin induced- peak 1 and phase 2 $[Ca^{2+}]_i$ rise in platelets from diabetic patients(control: black column; CBDMB: hatched column).

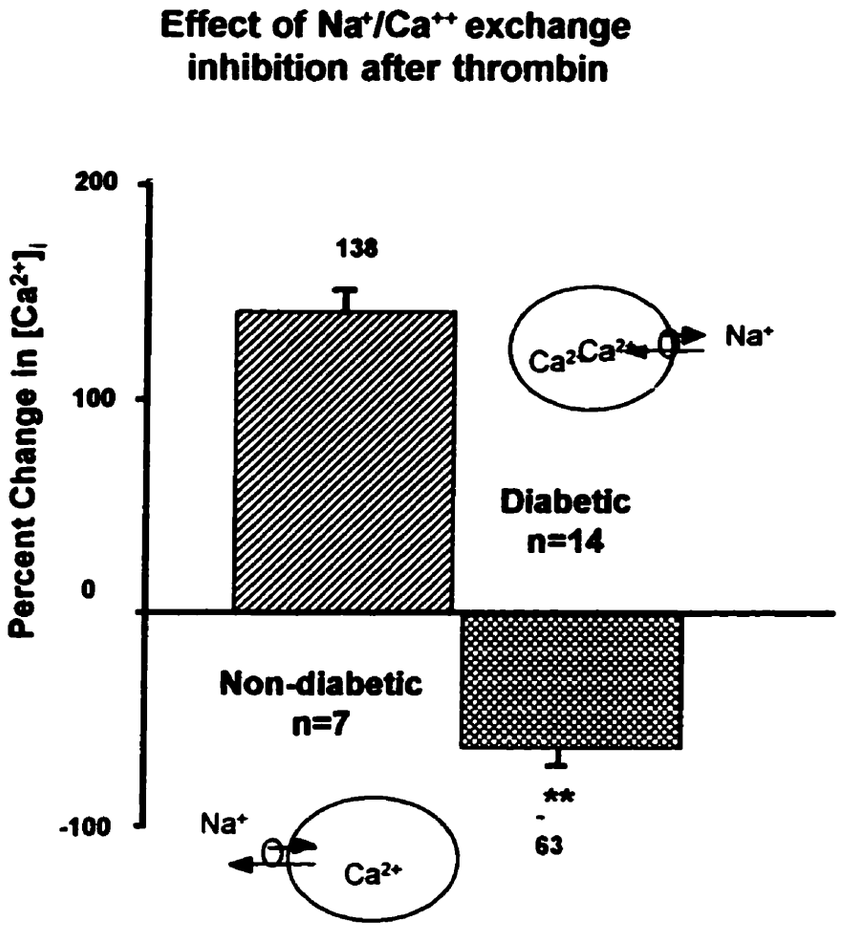


Figure 18

A comparison of the effects of CBDMB on thrombin-induced phase 2 $[Ca^{2+}]_i$ rise between platelets from nondiabetic subjects and diabetic patients. The vertical axis represents the percent change of thrombin-induced phase 2 $[Ca^{2+}]_i$ response after inhibition of Na^+-Ca^{2+}-exchanger by CBDMB. In nondiabetes the relative change after CBDMB was positive, whereas in diabetes the relative change after CBDMB was negative. This data suggests clearly that under thrombin activated condition the role of Na^+-Ca^{2+}-exchanger is different in platelets between normal subjects and diabetic patients.

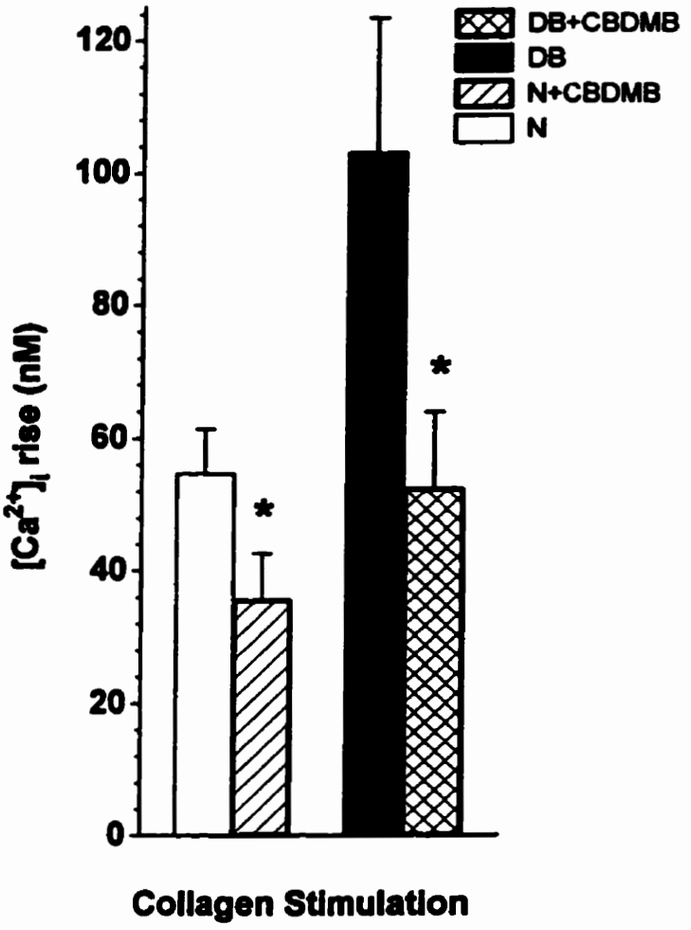


Figure 19

Effect of CBDMB on collagen-stimulated $[Ca^{2+}]_i$ response in platelets from normal subjects (n=10) and diabetic patients (n=8). In normals (N and N+CBDMB) CBDMB (2μM) decreased collagen (10μg/ml) induced platelet $[Ca^{2+}]_i$ rise indicating the Na^+-Ca^{2+}-exchanger works in the reverse mode in collagen stimulated condition. In diabetics (DB and DB+CBDMB) the same dose of CBDMB decreased collagen-induced $[Ca^{2+}]_i$ rise more suggesting the activity of reverse mode was increased in diabetes. Platelets were loaded with calcium-green and fura-red.

We then explored the role of the Na^+-Ca^{2+} exchanger in collagen-stimulated $[Ca^{2+}]_i$ rise by using CBDMB. As shown in Figure 19, collagen is different from thrombin: even in platelets from normal subjects, the addition of 2μM CBDMB decreased collagen-induced cytosolic $[Ca^{2+}]_i$ rise (from 54.69±6.63 to 35.65±7.02) suggesting that the Na^+-Ca^{2+} exchanger works in the reverse mode mediating Ca^{2+} influx. In diabetes, in spite of the higher $[Ca^{2+}]_i$ value after collagen, there was a greater decrease after CBDMB (from 107.75±23.00 to 56.14±12.59). These data indicate that after collagen stimulation, the reverse mode of the platelet Na^+-Ca^{2+} exchanger was activated and that the activity was further increased in the diabetic state.

Summarizing this part of the results, in platelets from diabetics the direction and activity of the Na^+-Ca^{2+} exchanger were altered. This may contribute, at least in part, to the deranged platelet calcium homeostasis. Under resting conditions, the forward mode of the Na^+-Ca^{2+} exchanger was decreased. In thrombin-stimulated condition, the forward mode activity was blunted and the reverse mode appeared to be operative. Under collagen- stimulated conditions, the activity of the reverse mode was enhanced.

Then we needed to determine how to relate this abnormal ion homeostasis to the enhanced cardiovascular disease in diabetes. In diabetes, there are many risk factors for cardiovascular disease such as hypertension, dislipidemia, etc. However diabetes by itself is an independent risk factor. Since platelets have an important role in the triggering and progression of diabetic vascular complications, and since data from clinical trials correlated hyperglycemia with the extent of diabetic complications, it is possible that there

is some link between hyperglycemia and platelet Ca^{2+} homeostasis and hyperactivity. To test this possibility, we determined the direct effect of elevated glucose on platelets from normal subjects in vitro.

PART III. THE IN VITRO EFFECT OF ELEVATED GLUCOSE ON PLATELETS FROM NORMAL SUBJECTS

In vivo, high concentrations of glucose in diabetic patients may affect platelets directly or indirectly. Hyperglycemia may act on platelets extracellularly or intracellularly. To investigate the direct effect of high glucose on platelets, we need to know first whether the glucose transport in platelets is insulin-sensitive.

GLUCOSE TRANSPORT IN PLATELETS

Glucose transport in platelets was studied by measuring the influx of 3-O-methyl-D-glucose (3OMG), a nonmetabolizable glucose analog, as described in the methods. The external 3OMG concentration was 0.6 mM. The influx was very rapid in the first 10 seconds of incubation, followed by a decreased rate. The uptake reached steady state at 10 minutes and 60 minutes (data not shown). This time course was consistent with the report of Kim (Kim et al., 1986). Based on this time course, we chose 2 points (10 seconds and 60 minutes) to study the effect of insulin on glucose transport. As shown in Figure 20, there was no significant difference in 3OMG influx in the control state and after

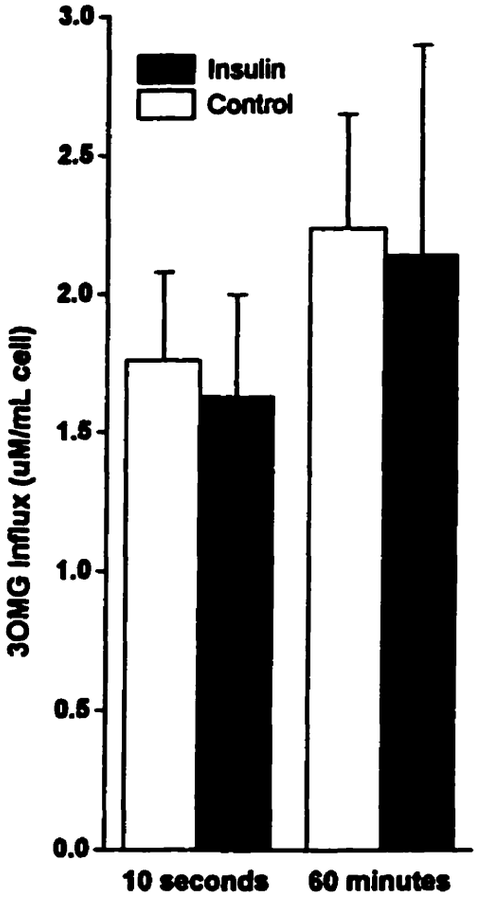


Figure 20

The insulin sensitivity of glucose transport in platelets. Glucose transport in platelets was determined by 3-O-methyl-glucose influx which was measured as described in the method. At 2 different time points (10 seconds and 60 minutes) the 3OMG influx was not significantly different in control (n=3, open column) and after 10μU/ml insulin pretreatment (n=3, grey column). The 3OMG was expressed as μM/ml platelets.

insulin (10μU/mL) treatment. Therefore, this result indicates that glucose transport in human platelets is not sensitive to insulin.

If the glucose transport is not sensitive to insulin, then intracellular glucose will accumulate in the presence of elevated extracellular glucose. It is therefore possible that this accumulation of intracellular glucose can pass through different metabolic pathways and affects platelet signal transduction mechanisms and function.

ACUTE EFFECT OF HYPERGLYCEMIA ON PLATELETS

To determine the acute effect of hyperglycemia dye-loaded platelets from the same normal subject were resuspended in 0 Ca^{2+} HEPES buffer containing 5 mM glucose or 45 mM glucose, respectively. 1 mM external calcium was then added and the agonist was applied 3 minutes later. As shown in Figure 21, both thrombin and collagen-induced $[Ca^{2+}]_i$ rise were not significantly different between the platelets in 5 mM glucose and 45 mM glucose medium. In addition the aggregation induced by thrombin and collagen was not different in these 2 groups.

THE PROLONGED EFFECT OF HYPERGLYCEMIA ON PLATELETS

The prolonged effect of high glucose was studied by incubating PRP for 24 hrs at 37°C in different glucose concentrations. After incubation, the PRP went through the same isolation and loading procedure and $[Ca^{2+}]_i$ and aggregation were measured. As shown in Figure 22 prolonged exposure of platelets to elevated glucose (45 mM) markedly enhanced platelet $[Ca^{2+}]_i$ response to collagen compared to the control (5 mM

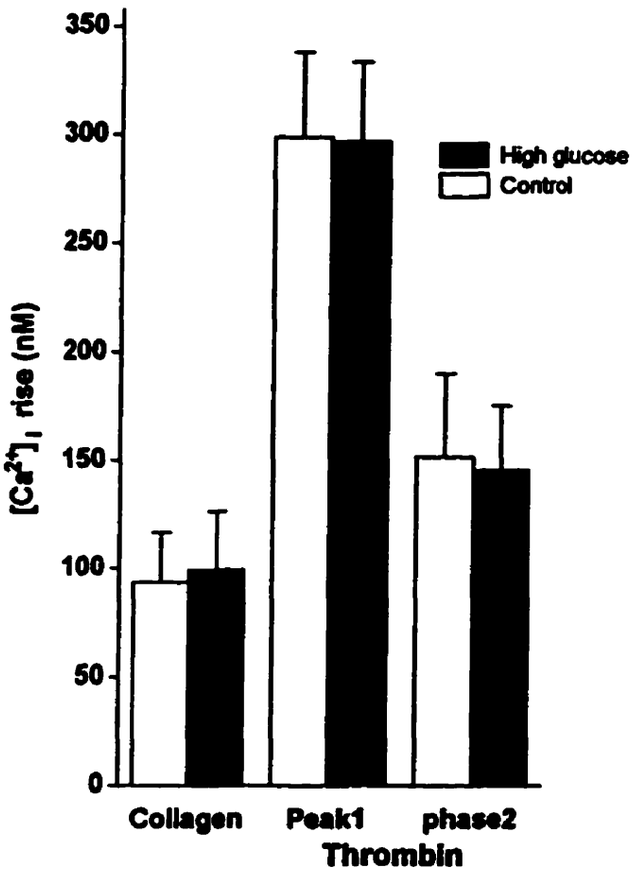


Figure 21

The acute effect of elevated glucose on thrombin (0.5 U/ml) and collagen(10 μg/ml)-induced $[Ca^{2+}]_i$ response. Platelets from normal subjects (n=4) were suspended in HEPES buffer containing 5 mM and 45 mM glucose respectively and 3 minutes later the agonist was added and the cytosolic $[Ca^{2+}]_i$ changes were measured. There was no significant difference in both thrombin and collagen-stimulated $[Ca^{2+}]_i$ rise between the 5mM and 45 mM glucose medium. Platelets were loaded with calcium-green, fura-red.

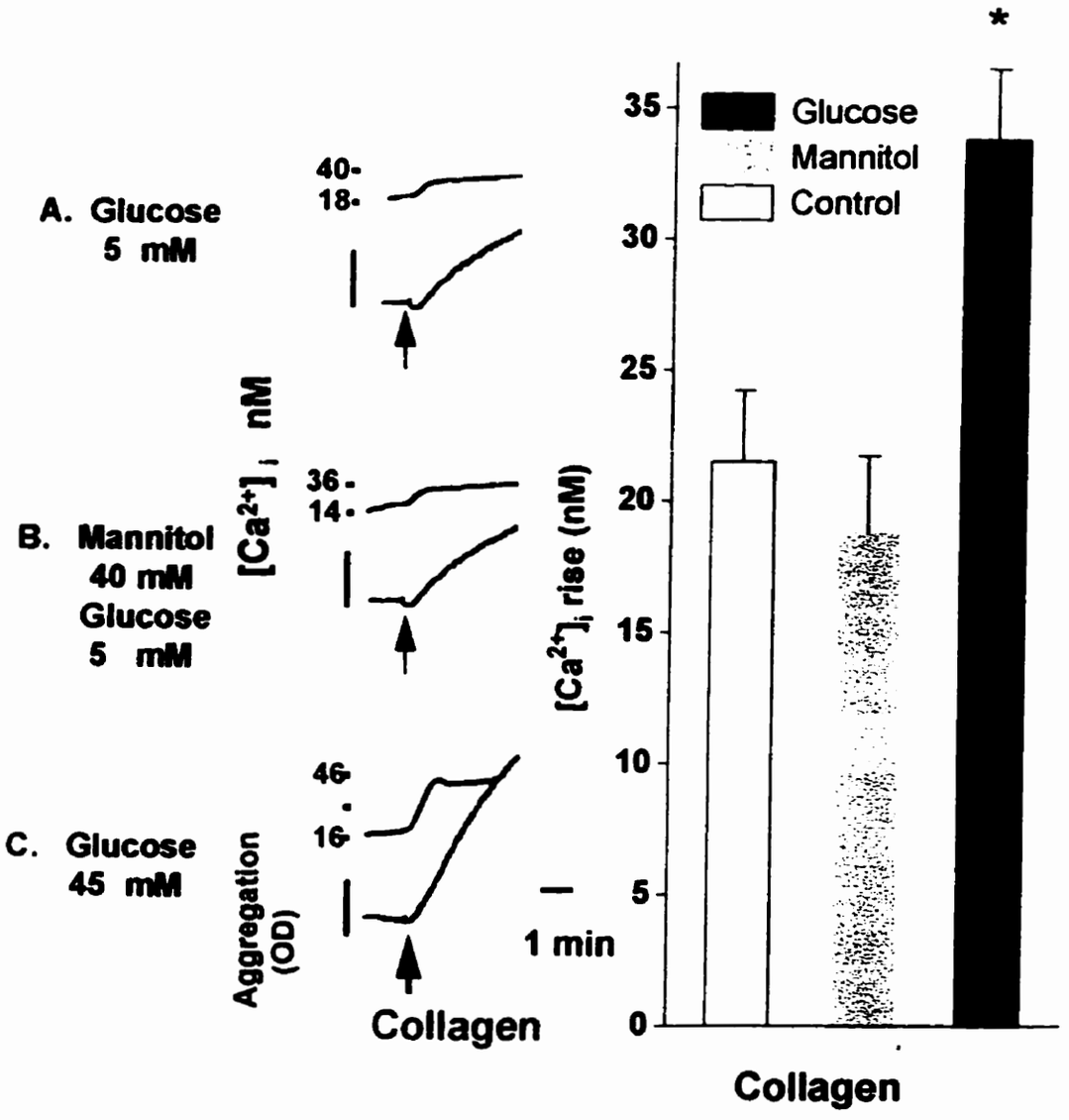


Figure 22

Effect of prolonged exposure of normal platelets with elevated glucose on collagen-induced $[Ca^{2+}]_i$ response and aggregation. PRP which was isolated from normal subjects was divided and incubated in three different conditions at 37°C as described in methods. A. 5 mM glucose(control); B. 5 mM + 40 mM mannitol(osmotic control); C. 45 mM glucose (high glucose). 24 hour later the platelets were loaded with calcium green and fura-red and platelet $[Ca^{2+}]_i$ and aggregation were monitored. Left: a typical trace showing the effect of high glucose on platelet $[Ca^{2+}]_i$ and aggregation. Right: summarized data from 6 experiments.

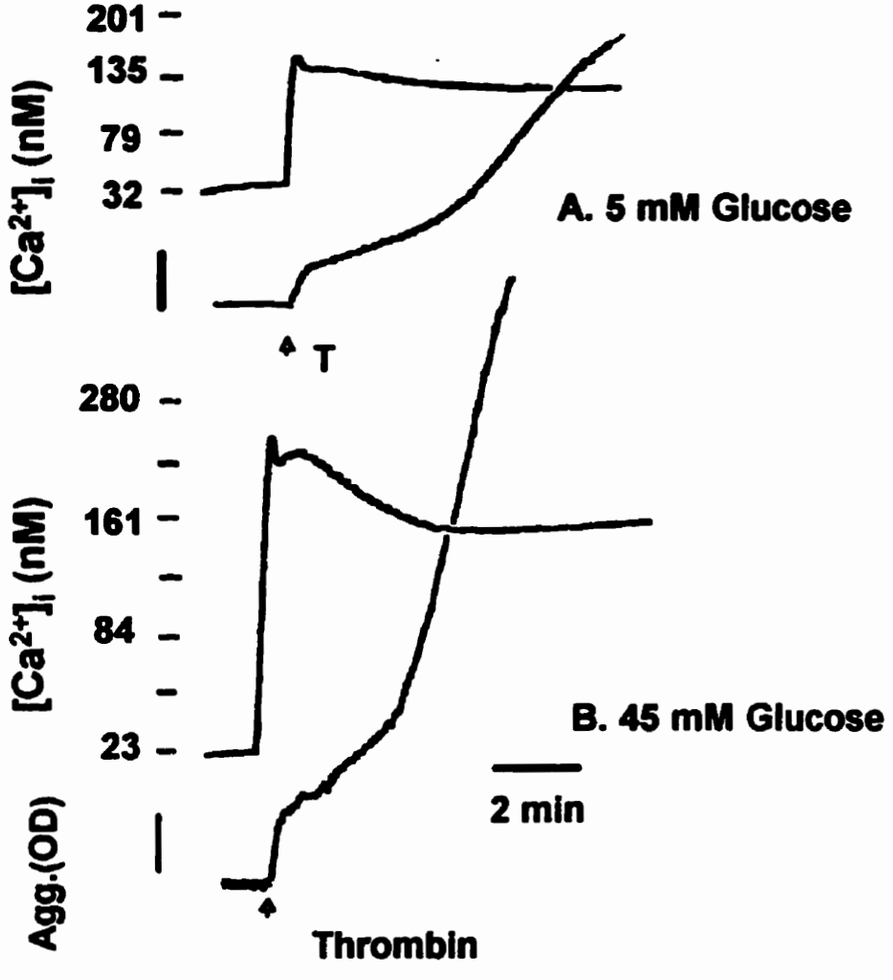


Figure 23

A typical trace of the effect of prolonged incubation of platelets with high glucose on thrombin-induced $[Ca^{2+}]_i$ and aggregation. PRP from normal subjects was incubated at 37°C for 24 hr in 2 concentrations of glucose: A. 5 mM glucose (control). B. 45 mM glucose (high glucose). Then platelets were loaded with calcium green and fura-red. T indicates thrombin (0.5U/mL).

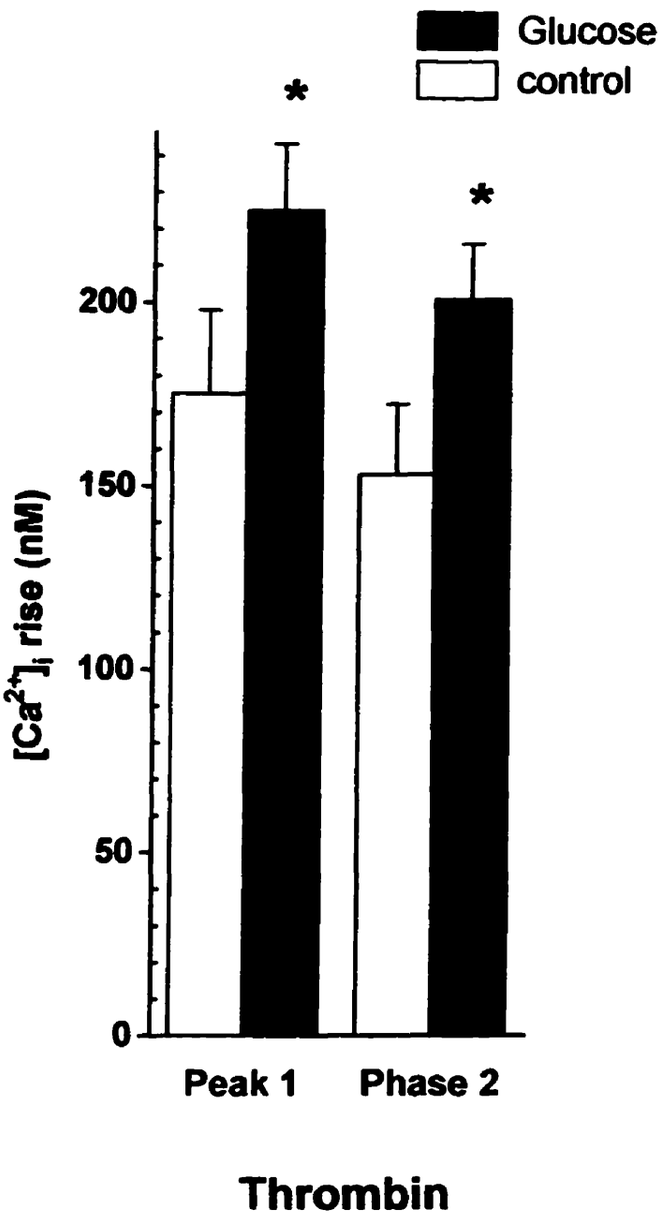


Figure 24

Effects of prolonged incubation of platelets with high glucose on thrombin-induced $[Ca^{2+}]_i$ response. PRP from normal subjects was incubated at 37°C for 24 hr in 2 concentrations of glucose: Control (5 mM glucose, open bar), High Glucose (45 mM glucose, black bar). Then platelets were loaded with calcium green and fura-red. Thrombin induced peak1 and phase 2 $[Ca^{2+}]_i$ response were higher in platelets exposed to high glucose compared to that in control. • $P<0.05$ n=5.

glucose). Similarly, the platelet response to thrombin was enhanced after high glucose incubation (Figure 23, 24). The agonist-induced platelet aggregation was also increased after 24 hour exposure to high glucose. There were no significant differences in platelet $[Ca^{2+}]_i$ and aggregation between control and high mannitol which was used to rule out the osmolar effect of high sugar concentration..

POSSIBLE MECHANISMS FOR THE EFFECT OF HYPERGLYCEMIA

Since we found that the platelet Na^+-Ca^{2+} exchanger is different in diabetes, it was of interest to know if the exchanger was also altered in hyperglycemia in vitro. Figure 25 shows the effect of CBDMB under the hyperglycemic condition. Platelets from normal subjects were first exposed to hyperglycemia for 24 hr then pretreated with CBDMB. Similar to the results seen in platelets from diabetic patients the thrombin-induced $[Ca^{2+}]_i$ response was significantly inhibited by CBDMB. These data suggest that the Na^+-Ca^{2+} exchanger played a role in the enhanced-thrombin-induced $[Ca^{2+}]_i$ response induced by hyperglycemia.

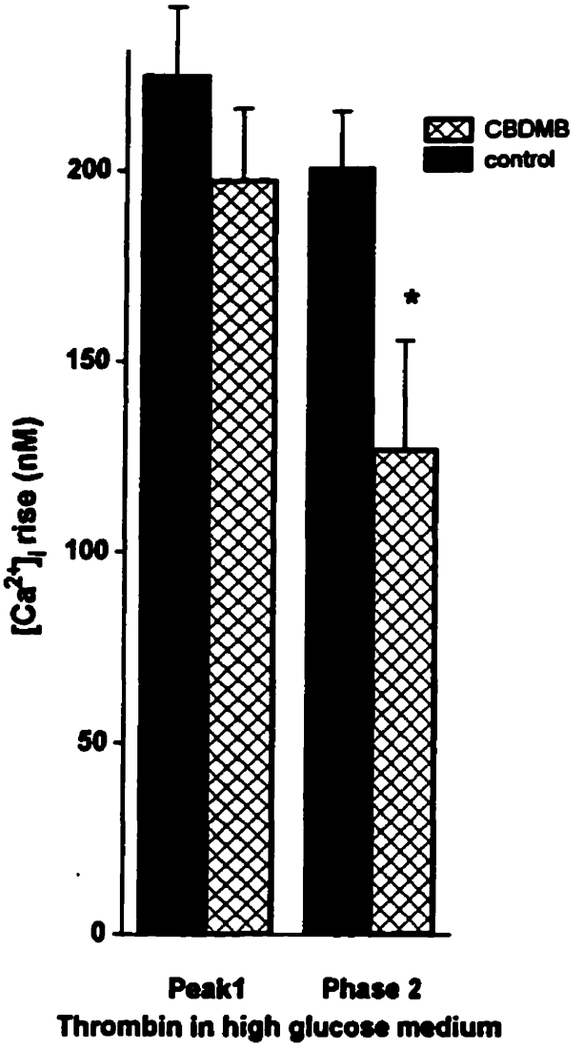


Figure 25

Inhibitory effect of CBDMB on thrombin-induced $[Ca^{2+}]_i$ response in platelets exposed with high glucose for 24 hours. In platelets preincubated with 45 mM glucose for 24 h at 37°C the increased platelet $[Ca^{2+}]_i$ was inhibited by CBDMB. Before CBDMB: black bar. After CBDMB: hatched bar. *$p<0.05$, n=5.

DISCUSSION

There are 3 major findings in this thesis. The first finding confirmed that in poorly-controlled diabetic patients, platelet cytosolic free calcium concentrations were increased under basal conditions and after stimulation by thrombin and collagen, compared to those in normal subjects. The second result determined the role of the Na^+-Ca^{2+} exchanger in regulating platelet Ca^{2+} homeostasis in normal subjects and found that in diabetes, the direction and activity of the platelet Na^+-Ca^{2+} exchanger are altered which may be one of the mechanisms for the increased cytosolic calcium and hyperactivity. In the third part we found that prolonged hyperglycemia in vitro can mimic the platelet calcium abnormality and hyperactivity seen in diabetic patients, suggesting that hyperglycemia per se may be one of the factors causing platelet hyperactivity.

PART I. ABNORMAL PLATELET CALCIUM HOMEOSTASIS IN DIABETES MELLITUS

PLATELET BASAL $[Ca^{2+}]_i$ IN DIABETIC PATIENTS

Fluorescent Ca^{2+} measurement is a sensitive tool in the study of the behavior of platelets in both normal and pathological conditions. The Ca^{2+}-sensitive fluorescence

indicator, quin-2/AM, was first developed by Tsien (Tsien, 1980). It was applied to the evaluation of platelet $[Ca^{2+}]_i$ by Rink et al., (1982). In 1985 a new probe, fura-2, was developed which overcame some of the problems with quin-2 (Grynkiewicz et al., 1985). The fluorescence method enables continuous monitoring of the calcium signal during activation. It can be used in cells without disruption, has an easily detectable fluorescent signal, is sufficiently sensitive and has kinetics fast enough to follow Ca^{2+} transients that occur during platelet activation. Fura-2 is currently considered to be the best method for the study of $[Ca^{2+}]_i$ particularly for its high wavelength sensitivity to Ca^{2+}. In this part of study, the platelets were loaded with fura-2 and the resting $[Ca^{2+}]_i$ of gel-filtered platelets was evaluated to be in the range of 80 nM in normal subjects, which is similar to other reports from platelets (Rink and Sage, 1990). This result suggests acceptable reproducibility of our method. In our study, the basal platelet $[Ca^{2+}]_i$ value was higher in the diabetic group than in the normal controls.

At this time, the results dealing with platelet basal $[Ca^{2+}]_i$ in diabetes are conflicting. Some reports showed no difference of platelet basal $[Ca^{2+}]_i$ between type II diabetes and control. For example, Srivastava et al., (1994) studied 3 groups of subjects: 19 NIDDM patients prior to control of blood glucose, 11 NIDDM patients after control of blood glucose and 26 normal subjects. They found basal $[Ca^{2+}]_i$ levels were similar in all three groups. Ishii (1990; 1991) also failed to find a significant difference in basal $[Ca^{2+}]_i$ among healthy subjects (n=8), type II diabetic patients with increased platelet aggregation rates (n=6), and type II diabetic patients with normal platelet aggregation rates (n=7).

On the other hand, Yamaguchi et al., (1991) reported that basal $[Ca^{2+}]_i$ was definitely higher in the NIDDM group than in the healthy controls after serial measurements of platelet $[Ca^{2+}]_i$ in 167 type II diabetic patients for a period of 2 years. It was also shown by Tschope (1991) that in type II diabetics platelet $[Ca^{2+}]_i$ was increased under basal conditions and after stimulation by collagen (2 μg/ml). This difference was more pronounced in the presence of extracellular Ca^{2+} than in the absence of extracellular Ca^{2+}. Mazzanti (1990) studied 15 IDDM and 22 NIDDM patients who had poor metabolic control and they found that platelet basal $[Ca^{2+}]_i$ was significantly higher in diabetic patients compared with control subjects, whereas there were no differences between IDDM and NIDDM patients. Our data are in agreement with the findings that reported a difference.

Pellegatta et al., (1993) measured platelet $[Ca^{2+}]_i$ in 60 IDDM patients and they found that as a whole group there was no difference between control subjects and diabetic patients. However when the 60 IDDM patients were divided into subgroups based on the presence of complications and metabolic status, it was found that the patients with a poor metabolic control (HbA_{1c} >8%) had higher resting $[Ca^{2+}]_i$. The presence or absence of retinopathy did not modify resting $[Ca^{2+}]_i$. Recently, Caimi et al., (1995) studied 21 type II diabetic subjects with macrovascular complications who had good metabolic control. They observed no difference in platelet resting $[Ca^{2+}]_i$ compared to that in 20 normal subjects.

From these published data, and from our results, it appears that metabolic control is an important factor that determines the platelet basal $[Ca^{2+}]_i$ levels. There are no differences between the types of diabetes. The discrepancy of results from different reports may be related to the number of subjects and different methods.

AGONIST INDUCED-PLATELET CALCIUM RESPONSE IN DIABETIC PATIENTS

Our data showed that in the presence of external Ca^{2+}, the thrombin and collagen stimulated $[Ca^{2+}]_i$ responses were enhanced. During thrombin stimulation, the phase 2 response was higher in platelets from diabetes, whereas the peak 1 response was not different compared to controls. The collagen-induced $[Ca^{2+}]_i$ response was enhanced dose-dependently in platelets from diabetics. Platelets are hypersensitive to many agonists such as thrombin, collagen, ADP, PAF etc (Colwell et al., 1983; 1988). Thrombin and collagen are two representative examples. Thrombin is a strong, physiological platelet agonist and is most frequently used in platelet calcium-handling studies (Siess, 1989). Collagen is a more physiological platelet agonist because it is the first agonist to occur after vessel injury and after atherosclerosis plaque disruption. Our results indicate that platelet Ca^{2+} homeostasis in basal and stimulated states is deranged in diabetes. In platelets, there are multiple mechanisms regulating cytosolic calcium, such as Ca^{2+} release and reuptake into the intracellular store, Ca^{2+} influx and efflux across the plasma membrane.

PART II: MECHANISMS OF ABNORMAL PLATELET CALCIUM HOMEOSTASIS IN DIABETES

In the presence of external Ca^{2+}, most of the thrombin-induced initial and transient $[Ca^{2+}]_i$ spike (peak1) reflects changes in the discharge of Ca^{2+} from intracellular stores such as the dense tubular system (DTS), whereas the sustained plateau (phase 2) reflects Ca^{2+} transport across the plasma membrane. The platelet response to collagen mainly utilizes extracellular calcium for $[Ca^{2+}]_i$ rise. Tschope (1991) also showed that the increase of collagen-stimulated platelet $[Ca^{2+}]_i$ response in diabetes was more pronounced in the presence of extracellular Ca^{2+}. The above results imply that in platelets from diabetics, the abnormality mainly exists in the Ca^{2+} transport mechanism in the plasma membrane.

INTRACELLULAR CA^{2+} RELEASE VS CA^{2+} TRANSPORT ACROSS PLASMA MEMBRANE

In the absence of external Ca^{2+}, our data clearly indicate that the intracellular store release mechanism is not altered in platelets from diabetics, although the extrusion and/or sequestration mechanisms are impaired. The intracellular Ca^{2+} store size was estimated by the peak response to a maximum dose of ionomycin in the absence of extracellular Ca^{2+} and no significant difference was found between controls and diabetics. We did not use caffeine or ryanodine because in the preliminary study of Ishida et al., (1993), they did not find any effect of these agents on $[Ca^{2+}]_i$ in intact platelets. The amount of stored Ca^{2+}, as shown in this study, may represent dischargeable free Ca^{2+} in intracellular storage sites, but not the total (i.e bound) amount of intracellular Ca^{2+} store. Secondly, the thrombin-induced $[Ca^{2+}]_i$ rise in the absence of extracellular Ca^{2+} did not differ between the 2

groups, which indicates that thrombin-induced intracellular Ca^{2+} release is not changed. Thirdly, in the absence of extracellular Ca^{2+} after thrombin stimulation, the rate of the $[Ca^{2+}]_i$ returning to basal level was slower in platelets from diabetics compared to that in normal subjects. Since the rate of $[Ca^{2+}]_i$ recovery represents the processes of Ca^{2+} reuptake from the cytosol into internal stores or/and extrusion out of the platelets, it is implied that the reuptake or/and extrusion mechanisms are impaired in platelets from diabetic patients. Because the intracellular store size was not different in diabetics as shown in our data and by Ishii's (1990) data, we suggest that the Ca^{2+} reuptake mechanism may not be impaired in diabetes, but further studies are needed to clarify this point.

The results about platelet Ca^{2+} mobilization from intracellular Ca^{2+} stores in diabetes are controversial. Ishii et al., (1991) reported that thrombin-(0.025-0.1U/ml) induced Ca^{2+} release was significantly enhanced in type II diabetic patients with high platelet aggregability. They demonstrated that this increased Ca^{2+} release was not due to Ca^{2+} store contents, but due to increased phosphoinositide turnover (Ishii et al., 1990). Mazzanti et al., (1990) found that thrombin-(0.2 U/ml) and ADP-(3 μM) induced $[Ca^{2+}]_i$ was higher in type II diabetics. In the absence of extracellular Ca^{2+}, the difference disappeared, suggesting that the higher platelet $[Ca^{2+}]_i$ values observed in diabetes cannot be ascribed to increased intracellular stores. This discrepancy in results from different studies may arise from the different doses of thrombin used or from racial differences in the diabetic patient population. In ours and in Mazzanti's results, 0.2U/ml and 0.5U/ml of thrombin were used and no difference was found in thrombin-induced intracellular Ca^{2+}

release. In Ishii's study, <0.1U/ml thrombin was used and they found a difference between diabetics and controls.

Our data suggest that the mechanisms for the abnormality mainly exist in the Ca^{2+} influx and extrusion across the plasma membrane. This conclusion is consistent with Bergh's (1988) results that were obtained using a ^{45}Ca method. However, the underlying mechanisms for this increased Ca^{2+} influx and decreased Ca^{2+} efflux are not clear. The next part of this thesis identified part of this mechanism.

SODIUM CALCIUM EXCHANGER

In platelets, the detailed mechanisms behind Ca^{2+} influx stimulated by thrombin and collagen are not fully understood. The possible pathways for Ca^{2+} entry could be receptor-operated channel (ROC), voltage-operated channel (VOC), store-depleted Ca^{2+} influx pathway as well as a Na^{+}-Ca^{2+} exchanger. The existence of VOC on platelets is controversial (Doyle and Rugg, 1985) and it was shown that Ca^{2+} channel blockers had no effect on platelet aggregation in type II diabetic patients (Klauser et al., 1990). ROC and store-depleted Ca^{2+} influx pathway are the subject of intensive research at the moment and have not been well characterized in platelets under thrombin and collagen-stimulated conditions.

The mechanisms of Ca^{2+} extrusion in platelets include the Ca^{2+}-ATPase and the Na^{+}-Ca^{2+} exchanger. It has been reported that in both type I and type II diabetic patients the platelet plasma membrane Ca^{2+}-ATPase activity was higher compared with that of

control subjects and there was a positive correlation between the platelet $[Ca^{2+}]_i$ and the increase of Ca^{2+}-ATPase activity (Mazzanti et al., 1990). The increase of Ca^{2+}-ATPase activity may be a compensatory mechanism for the increased $[Ca^{2+}]_i$ in diabetes. It is possible that the impaired mechanism for the decreased Ca^{2+} efflux observed in platelets from diabetes may be due to the Na^+-Ca^{2+} exchanger. The Na^+-Ca^{2+} exchanger is a reversible carrier mechanism that can mediate the transport of Ca^{2+} across the plasma membrane in 2 different directions depending upon the conditions. It is possible that the Na^+-Ca^{2+} exchanger may work in the reverse mode contributing to the enhanced Ca^{2+} influx in platelets from diabetes. Before studying the pathological role of the Na^+-Ca^{2+} exchanger we first investigated its physiological role.

Platelet Na^+-Ca^{2+} exchanger in normal subjects

We studied the physiological role of the Na^+-Ca^{2+} exchanger by 3 different approaches. First, the effect of reducing the transmembrane Na^+ gradient on platelet $[Ca^{2+}]_i$ in the resting state and after thrombin stimulation was investigated. If the Na^+-Ca^{2+} exchanger plays a role in regulating platelet calcium homeostasis, reducing the transmembrane Na^+ gradient should induce some changes in platelet $[Ca^{2+}]_i$; if an increase in $[Ca^{2+}]_i$ was seen it would indicate that the exchanger mediates Ca^{2+} efflux. There are 2 ways of reducing the transmembrane Na^+ gradient. One method is to decrease the extracellular Na^+ concentration in the medium (Na^+ can be replaced by iso-molar N-methyl-D-glucamine or sucrose or choline). A second method is to increase the intracellular Na^+ concentration by using either the Na^+-K^+-ATPase inhibitor, ouabain, or

by using the Na^+ ionophore, monensin. In our study, we used NMDG to partially replace extracellular Na^+, and in combination with ouabain to reduce the transmembrane Na^+ gradient. When the extracellular Na^+ was decreased to 28mM, basal $[Ca^{2+}]_i$ level and thrombin-induced phase 2 $[Ca^{2+}]_i$ response increased. Peak 1 of the thrombin response was not changed. When the thrombin response was studied in the absence of extracellular Ca^{2+}, we found that thrombin-induced Ca^{2+} release was not affected, whereas the cytosolic Ca^{2+} recovery process was slowed in 28 mM Na^+ medium. We also excluded the possibility that NMDG contained in the 28 mM Na^+ medium might have had non-specific effects on platelet $[Ca^{2+}]_i$ by comparing the effect of NMDG and sucrose in the same batch of platelets.

In our experiments, ouabain treatment alone had no effect on basal and thrombin-stimulated $[Ca^{2+}]_i$ response. The effect of ouabain can only be seen by a combination of low Na^+ and ouabain. Scheffer and Blaustein (1989) obtained similar results. The reason for this result could be that a marked effect of ouabain on platelet Ca^{2+} homeostasis can only be demonstrated after prolonged incubation (Roevens and Courcelles, 1990) since the influx of Na^+ down its concentration gradient by passive diffusion is the most likely limiting step in this phenomenon. Ishida et al.(1993) measured platelet $[Na^+]_i$ and found that ouabain increases $[Na^+]_i$ in a time dependent manner. Platelet $[Na^+]_i$ started to increase at 5 min following the addition of 0.1mM ouabain and reached a high level after 60 min. Resting and thrombin-induced $[Ca^{2+}]_i$ started to increase after 20 min. In our experiments 0.1 mM ouabain was applied for only 2 min prior to the addition of thrombin. It is thus possible that this duration is not sufficient for ouabain to increase $[Na^+]_i$.

Monensin was used to increase $[Na^+]_i$ by Valant et al., (1992) and they found that after pretreating platelets with monensin, there was a significant increase in $[Ca^{2+}]_i$.

Taken together, reducing the transmembrane Na^+ gradient either by a decrease in extracellular Na^+ or in combination with an increase in intracellular $[Na^+]_i$ can increase platelet cytosolic Ca^{2+} concentration, confirming the conclusion from the literature that the Na^+-Ca^{2+} exchanger exists in platelet plasma membrane. Further this suggests that the role of the Na^+-Ca^{2+} exchanger is to mediate Ca^{2+} efflux (forward mode) in resting state and after thrombin stimulation.

Secondly, the effects of specific inhibitors of the Na^+-Ca^{2+} exchanger were studied. Although the Na^+-Ca^{2+} exchanger has been shown to exist in many cell types, the physiological role of this exchanger has not been fully understood mainly because of the lack of specific and effective inhibitors in intact cells. With the availability of amiloride analogues that have different degrees of specificity and potency for inhibiting various Na^+-dependent processes (Kleyman and Cragoe, 1988), it is now possible to investigate the physiological role of the Na^+-Ca^{2+} exchanger.

Analogues of amiloride vary greatly in their potency and specificity to inhibit the Na^+ channel, the Na^+-H^+ exchanger and Na^+-Ca^{2+} exchanger activities. Phenamil specifically inhibits epithelial Na^+ channels and EIPA inhibits the Na^+-H^+ exchanger. For the Na^+-Ca^{2+} exchanger CBDMB is the most specific and potent one (K_i for the Na^+-Ca^{2+} exchanger 7.3μM, K_i for the Na^+-H^+ antiport, >500μM), although DCB (3'4'-

dichlorobenzamil, K_i for the Na^+-Ca^{2+} exchanger 30μM, K_i for the Na^+-H^+ antiport >400μM) has been used in many studies.

CBDMB has been screened for its ability to inhibit the Na^+-Ca^{2+} exchanger in rat anterior pituitary, bovine brain, cardiovascular tissues (Brown et al., 1991), intact lymphocytes (Wacholtz et al., 1992, 1993), basophil (Smith et al., 1992) and rat adrenal glomerulosal cells (Spat et al., 1989). In this study we tested the specificity of CBDMB in intact platelets by using the Na^+-dependent decline in $[Ca^{2+}]_i$ as the forward mode of the Na^+-Ca^{2+} exchanger. We found CBDMB effectively inhibits the platelet Na^+-Ca^{2+} exchanger in the μM range, consistent with the K_i value reported in other tissues. At this concentration range CBDMB had no effect on Na^+-H^+ exchange. It did not inhibit potassium or calcium channels (unpublished observation by Guia and Bose).

Whenever CBDMB and other amiloride analogues were used in the experiments, platelets were loaded with Ca-green/fura-red. Because these compounds fluoresce when excited with ultraviolet light, the Ca^{2+}-sensitive fluorescent dyes that are excited by UV irradiation such as fura-2 and indo-1 can not be used (Kraut et al., 1993). The absolute $[Ca^{2+}]_i$ level measured by this loading was lower than that seen in fura-2 loading, but the relative $[Ca^{2+}]_i$ change agreed very well with that in fura-2 loaded platelets.

Our results demonstrated that after CBDMB treatment, resting $[Ca^{2+}]_i$ increased in the presence of 140 mM external Na^+, the peak 1 of thrombin-stimulated $[Ca^{2+}]_i$ response remained the same, whereas the phase 2 increased significantly. These results suggest that

in physiological conditions the role of the Na^+-Ca^{2+} exchanger is to contribute to Ca^{2+} extrusion in the resting and thrombin stimulated states.

Thirdly to further confirm the above results the effect of a more specific and effective blocker of the Na^+-Ca^{2+} exchanger (XIP) was studied. XIP is a synthetic peptide that corresponds to residues 219-238 of the cardiac Na^+-Ca^{2+} exchanger and acts on an intracellular site. It has been successfully used to determine the physiological role of the Na^+-Ca^{2+} exchanger in cardiac muscle (Kohmoto et al., 1994). In our study we found that XIP enhanced the thrombin induced $[Ca^{2+}]_i$ response, an observation consistent with the results obtained with CBDMB.

By using 3 different approaches we obtained consistent data that suggests that in platelets from normal subjects the Na^+-Ca^{2+} exchanger appears to function mostly in the forward mode and helps to extrude cytosolic Ca^{2+} out of the cell during both the resting state and in the thrombin-activated state.

Platelet Na^+-Ca^{2+} exchanger in diabetic patients

In platelets from diabetics our results indicate that in the resting state the activity of the forward mode of the Na^+-Ca^{2+} exchanger was depressed. After thrombin stimulation the forward mode of the Na^+-Ca^{2+} exchanger was reversed. And following collagen stimulation the activity of the reverse mode was enhanced.

In most cells and in most situations, the role of the Na^+-Ca^{2+} exchanger is to pump Ca^{2+} out of the cell (forward mode), however under some conditions the exchanger can

mediate Ca^{2+} influx (reverse mode). The expected directions of Na^+ and Ca^{2+} movements through a Na^+-Ca^{2+} exchanger are determined by the net electrochemical gradient for the exchanger. The net driving force for the exchange can be estimated by the difference between the membrane potential (V_m) and the equilibrium potential of the exchange (V_{NaCa}): $\Delta V=V_m-V_{NaCa}$. If $\Delta V<0$, the Na^+-Ca^{2+} exchanger will extrude Ca^{2+} out of the cell (forward mode). If $\Delta V>0$, the exchanger will extrude Na^+ out and let Ca^{2+} in (reverse mode). There are two types of Na^+-Ca^{2+} exchanger: the cardiac type in which the stoichiometry is $3Na^+$:1 Ca^{2+} and the rod type, in which the stoichiometry is $4Na^+$:$1Ca^{2+}$, $1K^+$. The equilibrium potentials for these two types of exchangers are : $V_{NaCa}=3V_{Na}-2V_{Ca}$ and $V_{NaCa}=4V_{Na}-2V_{Ca}-V_K$ respectively. V_{Na} ,V_{Ca} and V_K are the equilibrium potential for Na^+, Ca^{2+} and K^+ respectively: $V_{Na}=(RT/F)\ln([Na]_o/[Na]_i)$. $V_{Ca}=(RT/2F)\ln([Ca]_o/[Ca]_i)$. $V_K=(RT/F)\ln([K^+]_o/[K^+]_i)$. R, T, and F are the gas constant, absolute temperature, and Faraday's number, respectively. In platelets whether the Na^+-Ca^{2+} exchanger is the cardiac or rod type is controversial. Both types are electrogenic. Therefore the net flux of Ca^{2+} and the rate of activity of the Na^+-Ca^{2+} exchanger can be affected by the transmembrane Na^+ gradient, Ca^{2+} gradient and membrane potential. In addition the Na^+-Ca^{2+} exchanger can be modulated by many factors, one of them is phosphorylation which has been shown to play an important role in modulating the Na^+-Ca^{2+} exchanger in VSMC (Shigekawa et al., 1996).

The altered Na^+-Ca^{2+} exchanger observed in the present study can be explained in two ways. One explanation is the expression of the exchanger protein is decreased in the diabetic state. However the data showing that the activity of the reverse mode is

increased makes this less likely. Another possibility is altered kinetic or regulatory properties of the exchanger in diabetes. This is quite possible since the direction and activity of Na^+-Ca^{2+} exchanger are determined by the Na^+ gradient, Ca^{2+} gradient, membrane potential and phosphorylation and in the diabetic state, all of these factors could be abnormal. One candidate is an increase of platelet cytosolic Na^+ in diabetes. It has been shown in both type I and type II diabetes that the platelet Na^+-K^+-ATPase activity is lower compared with that of control subjects and furthermore that the Na^+-K^+-ATPase activity is inversely related to platelet $[Ca^{2+}]_i$ (Mazzanti et al., 1990). This is supportive of our results, because a decrease of Na^+-K^+-ATPase activity can produce an increase in cytosolic Na^+ that would decrease the activity of the forward mode as seen in resting state. Following thrombin stimulation there may be more depolarization or changes in the phosphorylation state in diabetes which, in combination with increased cytosolic Na^+ concentration, can shift the exchanger to the reverse mode.

The reverse mode of the Na^+-Ca^{2+} exchanger has been reported in other disease states. For example during anoxia/ischemia in the CNS, most of the Ca^{2+} influx in white matter has been shown to be mediated by reverse mode of the Na^+-Ca^{2+} exchanger (Stys and Steffensen, 1996). In physiological conditions Na^+-Ca^{2+} exchanger has been described to mediate Ca^{2+} entry in cardiac cells (Leblanc and Hume, 1990), lymphocytes (Balasurbramanyam et al., 1994) and neutrophil (Simchowitz and Cragoe, 1988) after some form of stimulations. In this study we found that in platelets from normal subjects after collagen stimulation, the Na^+-Ca^{2+} exchanger works in reverse mode. The mechanism underlying this finding is not known. Tyrosine kinase has been shown to play

an important role in the signal transduction pathway of collagen (Blake et al., 1994). It is possible that tyrosine kinase can modulate the platelet Na^+-Ca^{2+} exchanger and shift it to work in reverse mode.

In conclusion, this part of the thesis, has demonstrated that the mechanisms for platelet abnormal Ca^{2+} homeostasis exist in the Ca^{2+} transport processes across the plasma membrane. Specifically, the direction and activity of the platelet Na^+-Ca^{2+} exchanger was altered which is responsible, at least in part, for the enhanced platelet Ca^{2+} influx, decreased Ca^{2+} efflux and increased $[Ca^{2+}]_i$ and hyperactivity in diabetes.

PART III: BIOCHEMICAL MECHANISMS OF PLATELET ABNORMALITIES IN DIABETES

EFFECT OF HYPERGLYCEMIA ON PLATELETS FROM NORMAL SUBJECTS

The third part of this thesis showed that hyperglycemia in vitro can affect platelet behavior from normal subjects suggesting that there is a relationship between glycemic control and platelet calcium homeostasis and function. The recent DCCT clinical trial (DCCT Research Group, 1993) determined the effect of tight glycemic control for 10 years in IDDM patients with retinopathy or nephropathy. They concluded that there is a correlation between the extent of glycemic control and the development of chronic complications. However the mechanism underlying this finding is not known. Our data

suggest that glucose per se can affect platelet Ca^{2+} homeostasis and aggregation and may represent one mechanism by which hyperglycemia leads to diabetic complications.

We first found that exposure of normal platelets to a pathological concentration of glucose (45 mM) for 3 minutes had no effect on platelets, in agreement with the results of Pellegatta (Pellegatta, 1993). In their study, the platelets were incubated at 37°C for 20 min in the medium containing different concentrations of glucose (from 1.68 mM to 56 mM). No difference in basal $[Ca^{2+}]_i$ and thrombin-induced $[Ca^{2+}]_i$ was found. Therefore, acute hyperglycemia is probably not harmful to platelet behavior.

Secondly, and of particular interest in this study, was that when the platelets were exposed for longer time periods (24hr, 37°C) thrombin and collagen-induced $[Ca^{2+}]_i$ responses and aggregation were enhanced. The effect of hyperglycemia was time-dependent and specific to glucose. No effect was found with an equiosmolar concentration of mannitol indicating the effect of glucose is not due to an osmotic effect. We chose the concentration of 45 mM based on published data showing that this concentration of glucose could affect the function of cultured endothelial cells (Graier et al., 1993) and aortic strips (Tesfamariam et al., 1990) within a few hours. It was demonstrated (Cohen, 1995) that the effect of in vitro exposure of the arteries to high glucose is limited in time. When concentrations of 400 to 800 mg/dL (22.2 mM to 44.4 mM) glucose were used in vitro for 3 to 6 hours, impaired endothelium-dependent relaxations and increased prostanoid production were observed, similar to effects seen in

arteries from rabbits that had been diabetic for 6 weeks, with plasma glucose concentration of 300 mg% (16.7mM).

These data in platelets are consistent with reports using other cells. High glucose has been found to increase cytosolic calcium in normal VSMC (Barbagallo et al., 1995), human erythrocytes (Resnick, 1993), pancreatic beta cells (Berggren et al., 1991) and insulinoma cells (Hoenig and Sharp, 1986).

MECHANISMS FOR THE EFFECT OF HYPERGLYCEMIA

Despite all the observations that glucose itself can alter intracellular $[Ca^{2+}]_i$, the mechanisms by which glucose may modulates Ca^{2+} homeostasis remain unclear. The present study showed that CBDMB can inhibit the thrombin-induced $[Ca^{2+}]_i$ response in hyperglycemic medium suggesting that the Na^+-Ca^{2+} exchanger may mediate Ca^{2+} influx in the enhanced $[Ca^{2+}]_i$ response in hyperglycemia. At this point, however, the relationship between the hyperglycemia and the Na^+-Ca^{2+} exchanger in platelets is not known. In other tissues such as the aorta, there is evidence for high glucose-mediated inhibition of the Na^+-K^+-ATPase (Gupa et al., 1992). As in diabetic patients (in vivo chronic hyperglycemia) a decrease in Na^+-K^+-ATPase activity could produce an increase in Na^+ concentration which, in combination with possible changes in membrane potential and phosphorylation, may shift the Na^+-Ca^{2+} exchanger to the reverse mode, resulting in Ca^{2+} entry and elevated cytosolic Ca^{2+} concentration.

The mechanism by which hyperglycemia produces an inhibition of the Na^+-K^+-ATPase is not clear. In this study, we found that the glucose transporter in platelets is insulin-insensitive, by measuring 3OMG influx. By using an immunoblotting method, Craik et al., (1995) found that platelet glucose transport is the Glut3 (brain type), which is not insulin-sensitive. This finding suggests that elevated glucose in vitro can affect platelets by accumulation of intracellular glucose, because in those cell types in which glucose transport is independent of insulin, intracellular glucose levels will increase in the presence of extracellular hyperglycemia.

In vascular cells, intracellular high glucose could affect cellular function by 4 mechanisms as reviewed in the first section of this thesis. Whether or not these mechanisms also exist in platelets remain to be determined.

One possible mechanism is the sorbitol-polyol pathway. In some cells, glucose can be converted to sorbitol by aldose reductase. When the glucose level is normal intracellular sorbitol concentration is very low because the K_m of aldose reductase for glucose is high. When intracellular glucose level increases the intracellular level of sorbitol will increase, which could produce adverse effects. This pathway was postulated to be responsible for the Na^+-K^+-ATPase inhibition in nerve fibers, kidney and aorta in hyperglycemic diabetic patients (Greene et al., 1988a; Simmons and Winegrad, 1989), since the inhibition of Na^+-K^+-ATPase activity can be prevented by aldose reductase inhibitors and by raising plasma myo-inositol levels in diabetic animals (Simmons and Winegrad, 1989). It is suggested that hyperglycemia acts by an increased activity of the

polyol pathway to induce some alterations in myo-inositol metabolism, possibly by inhibiting myo-inositol uptake and then myo-inositol depletion causes a decrease of Na^+-K^+-ATPase activity (Greene et al., 1988b).

In platelets, increased polyol pathway activity may play an important role in platelet hyperaggregation in the development of diabetic neuropathy. Hara, et al.,(1995) reported that an aldose reductase inhibitor, TAT (5-(3-thienyl) tetrazol-1-yl) acetic acid monohydrate, reduced ADP-induced platelet hyperaggregation in streptozotocin-induced diabetic rats with neuropathy.

The second mechanism could be through nonenzymatic glycation. Nonenzymatic glycation is the process in which glucose chemically attaches to proteins without involvement of enzymes. The formed glycation products could accumulate inside insulin-independent cells and outside on cell membrane proteins, circulating proteins and structural proteins, thus contributing to the pathogenesis of diabetic vascular complications (Brownlee., 1988, 1994). For example, changes in the physical properties of lens crystallin by glycation may be involved in cataract formation in diabetics (Stevens et al., 1978). Glycation rate is a function of time and glucose concentration. Usually it takes a few days for the non-enzymatic glycation to take place. Platelets, like other insulin-independent cell types, are freely permeable to glucose. Therefore platelet proteins are potentially susceptible to nonenzymatic glycation under hyperglycemic conditions. It has been shown (Sampietro et al., 1986) that when the isolated platelet membrane proteins were incubated at 37^0C with glucose (5-80mM/L) most of the glucose was incorporated

within the first 24 h. Why the glycation reaction in platelet plasma membrane protein is so fast is not clear. The experimental variables such as temperature variation and bacterial contamination have been excluded in their study. Under the same experimental conditions, the glycation of albumin is a slower linear process within a 6 day period. In another study, incubation of platelet membranes in 16.1 mM glucose for 10 days at 37°C led to an increase in the extent of glycation of membrane proteins. Most of the changes occurred within the first 3 days of incubation (Winocour et al., 1992). In our preparation, the intact platelets were incubated at 37°C for 24 hr. It is therefore possible that a non-enzymatic glycation mechanism is involved. In the literature, there is one report that studied the relationship between the nonenzymatic glycation of platelet proteins to the altered platelet function in diabetic patients (Yatscoff et al., 1987). They found that platelets from diabetic patients had a greater extent of glycation but there was no relationship between glycation and aggregation. It was concluded that glycation may not be responsible for the functional changes in platelets seen in diabetics. However, in their study the platelets from diabetics did not show an increased platelet aggregation and thromboxane production. Whether or not nonenzymatic glycation is involved in platelet calcium abnormalities needs to be clarified.

Another possible pathway involves hyperglycemia induced-diacylglycerol (DAG) formation and protein kinase C (PKC) activation. Hyperglycemia in vitro and diabetes can increase DAG levels and PKC activation in vascular cells (Lee et al., 1989; Inoguchi et al., 1992). The increased PKC may have multiple effects on cellular function related to vascular complications (Shiba et al., 1993). However, the identification of 12 isoforms

of PKC has complicated the dissection of the activation. Recently, by using a specific inhibitor of the beta-2 isoform of PKC. Ishii et al., (1996) found that activation of this isoform of PKC is the key step in the mechanisms by which glucose triggers diabetic vascular complications.

In vascular cells, there is evidence that the inhibition of the Na^+-K^+-ATPase is due to an activation of PKC induced by hyperglycemia. When the beta-2 isoforms of PKC are activated by hyperglycemia intracellularly, the cPLA2 activity is enhanced which is associated with increased production of arachidonate and PGE_2 (Nishio et al., 1995). Since PGE_2 and arachidonate are potent inhibitors of Na^+-K^+-ATPase (Schwartzman et al., 1985), this may be the mechanism by which hyperglycemia inhibits Na^+-K^+-ATPase. It was further demonstrated that PKC and cPLA2 inhibitors can normalize the inhibitory effect of hyperglycemia on Na^+-K^+-ATPase activities. Interestingly, in platelets the activity of the beta isoform of PKC is increased in IDDM but the relevance to diabetic complications is not clear (Bastyr and Lu, 1993).

In addition to affecting platelet $[Ca^{2+}]_i$, we found that prolonged exposure of platelets to high glucose could increase platelet aggregation. Similar data have been reported in other aspects of platelet function. Platelet adhesion has been shown to be increased by oral or intravenous glucose administration or by in vitro glucose addition (Bridges et al., 1965). Glucose added to samples of whole blood potentiates spontaneous platelet aggregation (SPA) (Loesche et al., 1988). It was suggested that this increased SPA is caused by liberation of ADP from red blood cells: because the effect of glucose on

SPA in whole blood was diminished by apyrase (an enzyme that removes ADP from blood plasma). Glucose was also found to increase the mechanical and osmotic fragility of RBC (Loesche et al., 1990).

In vivo, there is a lot of evidence demonstrating platelet hyperactivity in diabetic patients and that hyperactivity is correlated with the metabolic state. Some studies showed that after control of blood glucose in NIDDM patients, agonists such as ADP and arachidonic acid-stimulated platelet hyperaggregation and other biochemical parameters returned to normal (Srivastava et al., 1994). These in vitro effects of high glucose and studies in diabetic patients suggest that blood the glucose level is an important factor that determines platelet activity.

In conclusion, this part of the study indicates that prolonged hyperglycemia in vitro can induce abnormal Na^{+}-Ca^{2+} exchange activity, Ca^{2+} homeostasis and hyperactivity in platelets from diabetic patients suggesting that hyperglycemia per se is one of the factors which cause platelet abnormalities.

PART IV: GENERAL DISCUSSION

With the increased incidence of diabetes mellitus and the fact that the vascular complications are the most common cause of morbidity and mortality in diabetic patients, it is becoming more and more important to find out an effective preventive and therapeutic regimen for diabetic complications. As a result, the investigations about the pathogenesis of microangiopathy and the increased atherosclerosis in diabetes have been an interesting

research area for many years. Many hypotheses have been developed to explain the pathogenesis of diabetic vascular complications. One of these is that abnormal platelet activity is associated with both diabetic micro and macroangiopathy. It has been found that there is a prothrombotic state in diabetes including increased intravascular thrombin generation, reduced fibrinolytic potential and hyperactive platelets. Among these, hyperactive platelets is a main determinant of the thrombotic state in diabetes because it has been shown that acute thrombotic events in the arterial circulation are predominantly triggered by platelets. Activated platelets could contribute to diabetic micro and macroangiopathy possibly by microembolization of the capillaries or by secreting constrictive, mitogenic, and oxidative substances resulting in local progression of preexisting vascular lesions. In addition, hyperactive platelets could be a trigger for the prognosis limiting the arterial thrombotic event.

Studies of platelet function in vitro indicate that platelet sensitivity to aggregating agents is enhanced in diabetes. Platelets are very good models for revealing the various abnormalities that could be relevant to the pathogenesis of vascular diseases since they are one of the few cell types that can be obtained readily from the blood of healthy human volunteers and patients. Moreover, platelets are a homogeneous pure cell suspension that can respond reliably and quantifiably to many agonists of physiological and pharmacological interest.

There are a lot of studies in the literature demonstrating platelet hyperactivity and such hyperactivity seems to be a universal phenomenon in diabetic patients. Platelet

hyperactivity has been found to exist in both humans and animals with diabetes mellitus, in type I and type II diabetes, in preclinical diabetes, clinical uncomplicated diabetes and in diabetes with vascular complications (Gensini et al., 1979). When we selected the diabetic patients in our study, we did not differentiate between the type of diabetes and the associated complications. Only the metabolic control level (Hb_{A1c}) was considered, because the overall object of this thesis was to determine the mechanisms of platelet hyperactivity in the diabetic state.

Since the platelet hyperactivity occurred also in washed platelets, the hyperactivity should result from a change in the properties of platelets themselves rather than from an alteration in the plasma and availability of blood-borne activating factors.

In general, changes at all levels of the platelet activation system could contribute to an enhanced functional response (aggregation) in patients with diabetes mellitus. For example amplified agonist-receptor coupling, increased thromboxane formation, increased alpha granule content, increased platelet volume, and increased numbers of glycoprotein receptors GPIb and GPIIb/IIIa could be involved in platelet hyperactivity.

In the literature, the platelet hyperactivity in diabetes has been attributed to increased platelet arachidonate metabolism leading to increased TXA_2 formation. However in clinical trials, aspirin had only limited effects. In experimental studies, it was found that platelets from diabetics remained hypersensitive in the presence of CP/CPK which removed ADP and of aspirin that inhibited the arachidonate pathway (Winocour, et al., 1986). These findings indicate that platelet hyperactivity in diabetes also operates

through a mechanism(s) that is (are) independent of released ADP and activation of the arachidonate pathway.

Abnormal intracellular Ca^{2+} concentration seems to be a common defect in both type I and type II diabetes. In diabetes abnormal Ca^{2+} homeostasis has been reported to exist in many cells such as cardiac muscle, aorta, mesenteric and coronary arteries, skeletal muscle, kidney, liver, red blood cells, lens, adipocytes in diabetic animals and in arteries, red blood cells and adipocytes in diabetic patients (Levy et al., 1994). Along with some reports in the literature, the first part of this thesis confirmed that there was also abnormal Ca^{2+} handling in platelets. In platelets increased aggregation, and thus an increased thrombotic tendency, as well as the release of humoral factors such as platelet-derived growth factors are all consequences of increased platelet cytosolic free calcium levels. Abnormal Ca^{2+} homeostasis is one of the nonarachidonate mechanisms responsible for platelet hyperactivity in diabetes.

There are multiple regulatory mechanisms for cytosolic free Ca^{2+} concentration in platelets and other cells. In diabetes the possible defect may involve one or more regulatory mechanisms. As reported in the second part of the thesis, in platelets from diabetics intracellular calcium release is not changed, but the defect exists in the Ca^{2+} transport mechanism across the plasma membrane. This is consistent with the report that Ca^{2+} influx was increased and Ca^{2+} efflux rate was decreased by using $^{45}Ca^{2+}$ method. Specifically we found that the direction and activity of the Na^{+}-Ca^{2+} exchanger was abnormal. The activity of the forward mode of the Na^{+}-Ca^{2+} exchanger was blunted while

the activity of reverse mode of Na^+-Ca^{2+} exchanger was increased. We did not measure Na^+-Ca^{2+} exchanger activity in isolated vesicles. We studied the Na^+-Ca^{2+} exchanger activity in intact platelets because this approach is more relevant to physiological and pathological conditions. In platelets from diabetic patients, it has been shown that activity of the Na^+-K^+-ATPase was decreased which is supportive of our results since a decrease in Na^+-K^+-ATPase activity would increase intracellular Na^+ concentration which can then decrease the activity of the forward mode of the Na^+-Ca^{2+} exchanger. In combination with other possible changes, such as depolarization and phosphorylation, the Na^+-Ca^{2+} exchanger would be shifted to the reverse mode contributing, at least in part, to the enhanced Ca^{2+} influx.

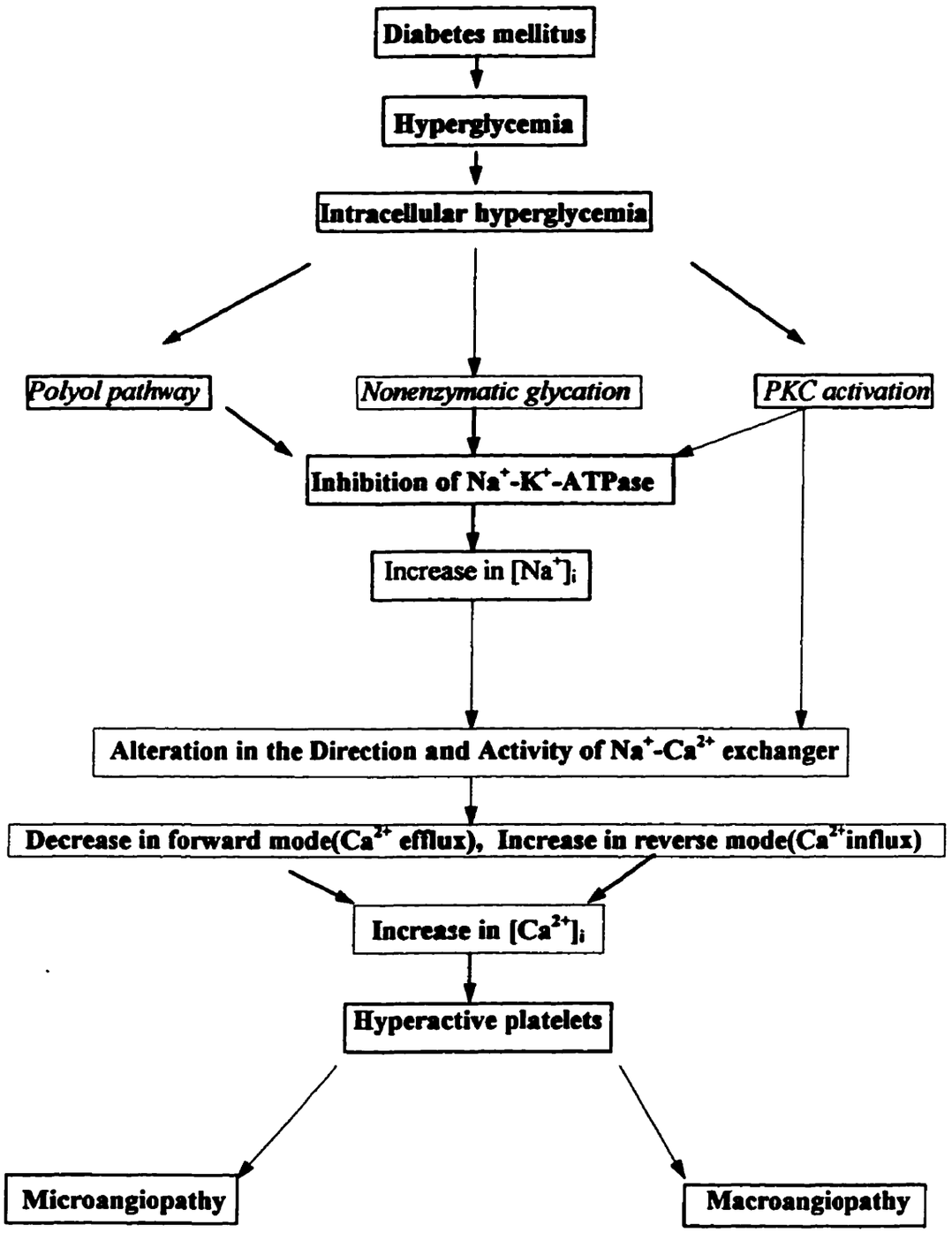
Interestingly, the activity of the platelet Ca^{2+}-ATPase has been reported to be increased in diabetes and this activity is correlated with the cytosolic $[Ca^{2+}]_i$ concentration. This could be a compensatory extrusion mechanism for the increased basal $[Ca^{2+}]_i$ and enhanced agonist-induced $[Ca^{2+}]_i$ response observed in platelets from diabetics.

The possible defect or primary mechanism for abnormal calcium homeostasis may be different in different tissues. For example, it has been found that the Ca^{2+}-ATPase activity is decreased in red blood cells in diabetes. The malfunction of this ATPase in diabetic patients may be genetically determined (Levy et al., 1989), but may also be secondary to deleterious diets such as a high-fat diet, which can change membrane

function (Storlien et al., 1987). Hyperglycemia-induced glycosylation of the Ca^{2+}-ATPase molecule can also decrease its activity (Flecha et al., 1990).

As described in the third part of this thesis, we identified that the abnormality of the Na^{+}-Ca^{2+} exchanger and calcium homeostasis in platelets is secondary to metabolic derangements in the diabetic state, most specifically, hyperglycemia. There are many metabolic derangements in diabetes such as hyperglycemia, hyperlipidemia, hypertension, obesity etc. Hyperglycemia is a metabolic change specific to diabetes, and is the major initiator of diabetic vascular complications. Our data suggest that platelets could be one of the mechanisms linking hyperglycemia to the diabetic complications.

The following model (see next page) illustrates the main scheme (shown as bold) of this thesis. Regarding how the metabolic factor of hyperglycemia affects platelet Ca^{2+} homeostasis and activity, there are 3 possible mechanisms (shown in italics in the model) which could represent the future directions of the work described in this thesis.


Diabetes mellitus
Hyperglycemia
Intracellular hyperglycemia
Polyol pathway
Nonenzymatic glycation
PKC activation
Inhibition of Na^+-K^+-ATPase
Increase in $[Na^+]_i$
Alteration in the Direction and Activity of Na^+-Ca^{2+} exchanger
Decrease in forward mode(Ca^{2+} efflux), Increase in reverse mode(Ca^{2+}influx)
Increase in $[Ca^{2+}]_i$
Hyperactive platelets
Microangiopathy
Macroangiopathy

CONCLUSIONS

Following is the summary of the major findings in this thesis:

•In metabolically uncontrolled diabetic patients, platelet calcium homeostasis is deranged: both basal $[Ca^{2+}]_i$ level and agonist (thrombin and collagen)-stimulated $[Ca^{2+}]_i$ responses are enhanced.

•The Ca^{2+} store content in DTS as well as thrombin-induced Ca^{2+} release is not changed in platelets from diabetics.

•In diabetes, the platelet abnormal Ca^{2+} homeostasis originates from a change in transmembrane Ca^{2+} movement.

• The Na^+-Ca^{2+} exchanger exists in the plasma membrane in platelets from normal subjects and appears to mainly function in the forward mode (Ca^{2+} efflux) in resting and thrombin-stimulated conditions.

•CBDMB (at μM range) is a very potent and specific inhibitor of the Na^+-Ca^{2+} exchanger in intact platelets and has no effect on the Na^+-H^+ exchanger.

- In platelets from diabetics the direction and activity of the Na^+-Ca^{2+} exchanger are altered, which is one of the mechanisms underlying the abnormal Ca^{2+} homeostasis in diabetes.

- In the resting state, the forward mode is blunted

- During thrombin stimulation, the forward mode is decreased and the reverse mode is activated, mediating Ca^{2+} influx

- During collagen stimulation, the reverse mode is increased.

•Glucose transport in platelets is not insulin-sensitive.

•Acute hyperglycemia is not harmful for platelet behavior.

•Exposure of normal platelets to hyperglycemia for a prolonged duration produces an enhancement of the platelet Ca^{2+} response to agonists and aggregation, which can be inhibited by CBDMB.

In diabetes, platelet Ca^{2+} homeostasis is abnormal and this is partly responsible for the platelet hyperactivity. The alteration in the direction and activity of the platelet plasma membrane Na^+-Ca^{2+} exchanger is one of the mechanisms for the abnormal Ca^{2+} homeostasis. These abnormalities observed in diabetic patients can be mimicked by the in vitro exposure of normal platelets to prolonged hyperglycemia, suggesting that the underlying biochemical mechanisms for these platelet abnormalities are secondary to the metabolic factor, hyperglycemia.

Based on the findings described in this thesis, potential drug development can be targeted at 2 sites to prevent the progression of diabetic complications. One approach is to block the pathway that leads to the increased platelet basal $[Ca^{2+}]_i$ and enhanced $[Ca^{2+}]_i$ response to agonists. The Na^+-Ca^{2+} exchanger is a good target. Another approach is to tightly control blood glucose levels in diabetic patients and to block the pathways linking hyperglycemia and platelets and other tissues. Three postulated mechanisms: aldose reductase, nonenzymatic glycation, and DAG-PKC can be targeted.

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