STUDIES ON THE EFFECT OF RAT C-REACTIVE PROTEIN ON PLATELET AGGREGATION AND SECRETION

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Studies on The Effect of Rat C-Reactive Protein on Platelet Aggregation and Secretion.

By

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Abstract

Rat C-reactive protein (CRP) is a member of a family of proteins, collectively termed C-reactive proteins, present in a variety of species, from simple invertebrates to humans. In this thesis, the effect of rat CRP on platelet function was examined. Rat CRP inhibited, in a dose-dependent manner, platelet aggregation and serotonin secretion using either ADP or thrombin as agonist. Inhibition of platelet aggregation induced by these agonists required the binding of rat CRP to platelets. This binding was specific, saturable, of high affinity and calcium-sensitive. The rat [^{125}I]CRP bound to platelets was displaced by either unlabelled rat CRP or phosphorylcholine. This binding was also inhibited by either rabbit CRP or phosphorylcholine.

Rat CRP inhibited platelet activating factor (PAF)-induced platelet aggregation. Results showed that the inhibition of PAF-induced platelet aggregation required the binding of rat CRP either to platelets or to PAF.

Platelet functional responses are mediated by the participation of platelet phospholipases in signal transduction pathways. Therefore the effect of rat CRP on agonist-induced platelet phospholipase activity was examined. The results confirmed the presence of phosphatidylcholine-specific phospholipase C (PC-PLC) in rat platelets. This platelet PC-PLC was inhibited by rat CRP in a dose-dependent manner. In contrast, rat CRP did not inhibit either phospholipase A_2 or phosphatidylinositol-specific phospholipase C activity. It was, therefore, concluded that if the formation of

phosphatidylcholine-derived diacylglycerol (DAG) by PC-PLC is involved in the regulation of platelet functional response, then the inhibition of DAG formation by rat CRP may be critical in the observed inhibition of platelet aggregation and serotonin secretion by rat CRP.

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Publications

Much of the work presented in this thesis has been published or submitted for publication as full papers. These papers are included in the following list of publications:

1) Randell, E., Mookerjea, S., and Nagpurkar, A. (1992) Inhibition of thrombin-induced platelet aggregation and secretion by Rat C-reactive protein: Possible involvement of phosphatidylcholine-specific phospholipase C. (Submitted to *J. Biol. Chem.*)

2) Randell, E., Mulye, H., Mookerjea, S., and Nagpurkar, A. (1992) Evidence for phosphatidylcholine hydrolysis by phospholipase C in rat platelets. *Biochim. Biophys. Acta.* **1124**, 273-278.

3) Randell, E., Mookerjea, S., and Nagpurkar, A. (1990) Interaction between rat phosphorylcholine-binding protein and platelet activating factor. *Biochem. Biophys. Res. Comm.* **167**, 444-449.

4) Randell, E., Mookerjea, S., and Nagpurkar, A. (1990) Binding of rat serum phosphorylcholine-binding protein to platelets. *Biochim. Biophys. Acta.* **1034**, 281-284.

5) Nagpurkar, A., Randell, E., Choudhury, S., and Mookerjea, S. (1988) Effect of rat phosphorylcholine-binding protein on platelet aggregation. *Biochim. Biophys. Acta.* **967**, 76-81.

Other Work:

1) Prabhakaran, V.M., and Randell, E. (1992) Study of carryover at lower end TSH concentrations by Delfia hTSH assay and Quatro pipetting station. (Submitted to *J. Clin. Biochem.*)

2) Omar, R., Randell, E., and Rahimtula, A. (1991) In vitro inhibition of rat platelet aggregation by Ochratoxin A. *J. Biochem. Toxicol.* **6**, 211-220.

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List of Abbreviations

ADP	Adenosine diphosphate
BSA	Bovine serum albumin
CPS	C-polysaccharide
CRP	C-reactive protein
DAG	1,2-Diacylglycerol
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
FP	Female protein
HDL	High density lipoprotein
HETE	12-Hydroxyeicosatetraenoic acid
HHT	12-Hydroxyheptadecatrienoic acid
HPLC	High performance liquid chromatography
IP_3	Inositol-1,4,5-triphosphate
LDL	Low density lipoprotein
LPC	Lysophosphatidylcholine
PA	Phosphatidic acid
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PC-PLC	Phosphatidylcholine-specific phospholipase C

PE..... Phosphatidylethanolamine

pI..... Isoelectric point

PI..... Phosphatidylinositide

PIP_2..... Phosphatidylinositol-4,5-bisphosphate

PI-PLC..... Phosphatidylinositol-specific phospholipase C

PKC..... Protein kinase C

PLC..... Phospholipase C

PLD..... Phospholipase D

PLA_2..... Phospholipase A_2

PRP..... Platelet rich plasma

PS..... Phosphatidylserine

TLC..... Thin layer chromatography

SAP..... Serum amyloid P-component

SDS..... Sodium dodecyl sulphate

VLDL..... Very low density lipoprotein

CHAPTER 1

PLATELETS AND THEIR FUNCTION

Platelets normally circulate in the blood stream in a resting state, showing little tendency to interact with each other or with blood vessel endothelium. Upon stimulation, however, platelets play a critical role in haemostasis through aggregatory reactions that could result in life threatening thrombotic events if not controlled. Mechanisms must, therefore, exist to limit and regulate platelet aggregatory response in order to maintain the vascular system without endangering life.

There is considerable evidence for the involvement of platelets in the pathogenesis of a wide variety of diseases such as thrombosis and atherosclerosis (Becker, 1991). Platelets contribute to normal haemostasis by adhering to exposed proteins in the subendothelial tissue following vascular damage. This is immediately followed by the recruitment of additional platelets in a process known as platelet aggregation, which results in formation of a platelet plug. The subsequent activation of the clotting cascade forms a network of fibrin which reinforces the aggregated platelets (Packham and Mustard, 1984). The degree of platelet involvement is dependent on their level of activation. Overactive platelets are implicated in the pathogenesis of many diseases including cardiac infarction and stroke (Rink and Hallam, 1984; Sullivan, 1984). However, the most convincing evidence for the role of platelets in coronary heart disease is a marked reduction of pathogenic events by

the use of aspirin which inhibits cycloxygenase activity (Weissman, 1991). Platelets also play an important role in acute inflammation. During the acute phase response, platelets have been observed to become activated and release their granule contents, which may contribute to the inflammatory process (Page, 1989).

Platelets can be activated by several physiological and non-physiological agonists, most of which are presumed to act on specific platelet membrane receptors (Colman, 1991). The platelets respond to these external stimuli with a series of cellular events that usually follow the same pattern: activation, shape change, aggregation and the secretion of substances from three types of granules (Holmsen, 1989). The interaction between a primary stimuli or agonist and its receptor causes rapid mobilization of intracellular second messengers, as well as formation of certain intercellular messengers. These signal molecules play a central role in mediating cellular biochemical reactions leading to platelet aggregation and secretion (Holmsen, 1989).

Section I. Platelet Origin and Structure

Platelets are non-nucleated, discoid cells that are 1 μm thick and 3 μm in diameter (Rink and Hallam, 1984). The platelet volume is roughly 1/13 the volume of red blood cells and platelets number about 2.5-6.0 x 10^8 per ml of circulating human blood. Ultrastructural features of platelets are shown in Fig. 1. The discoid shape of platelets is maintained by a circumferential band of long microtubules inside

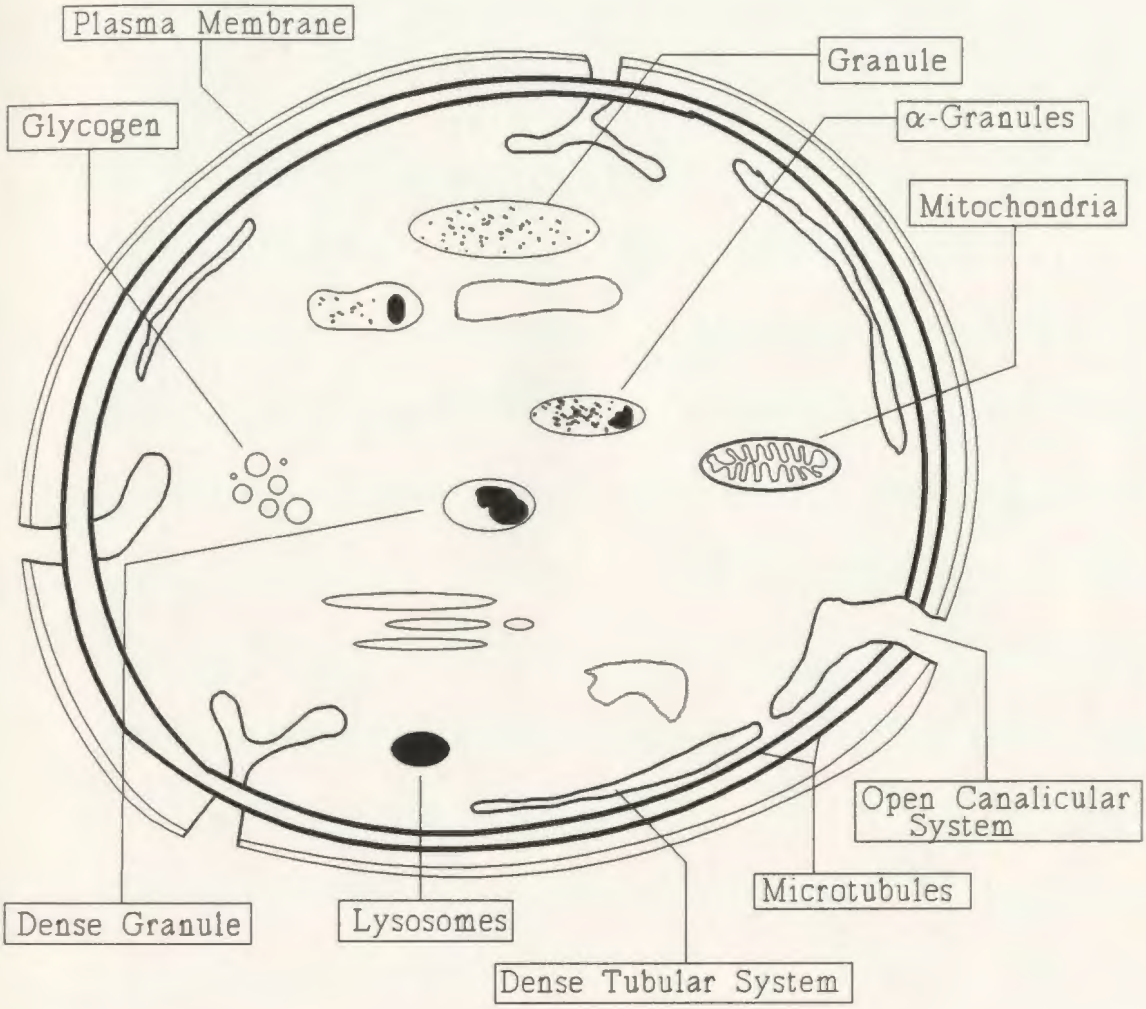


Fig. 1. **Ultrastructural features of platelets.** Diagram shows a typical platelet and sub-cellular components that are visible by electron microscopy (Adapted from White, 1984).

the external membrane which serve as a skeletal frame (White, 1987a&b). The cytoplasm contains a number of organelles including mitochondria, vacuoles, stored glycogen, and numerous open vesicles or membranous channels. These channels are connected to one another and to the surface of the platelet to form the open canalicular system (White, 1984), which provides access to the interior for plasma bound substances and an escape route for products released by activated platelets. Also localized in the cytoplasm is the dense tubular system, which, with the aid of Ca^{2+}-Mg^{2+} ATPase (Cutler et al., 1978), is the site of calcium sequestration and contains the enzymes responsible for prostaglandin synthesis (Gerrard et al., 1978).

Platelet cytoplasm contains several types of granules which can be distinguished on the basis of their contents (White, 1984). The most dense granule, appropriately called dense granules, contain the vasoactive amine serotonin, calcium and a non-metabolic pool of ADP and ATP (Stenberg and Bainton, 1987). The second type of granule, the α-granule, is less dense and more numerous than dense granules. These granules contain platelet factor 4, factor 5, factor 7, platelet-derived growth factor, von Willebrand factor, thrombospondin, ß-thromboglobulin, fibrinogen and fibronectin (White, 1984; Stenberg and Bainton, 1987). The third type of granule are lysosomes which contain enzymes such as acid phosphatase, N-acetyl-glucosaminidase and β-glucuronidase, required for degradation of proteins and carbohydrate (Stenberg and Bainton, 1987). All three types of granules may be emptied into the extracelluar environment on platelet activation.

The plasma membrane of platelets contain a number of glycoproteins which are important to platelet function. In fact, the platelet glycocalyx is much thicker and more concentrated than that covering the outer surface of other blood cells (White, 1984). While some of these glycoproteins are responsible for interactions of platelets with external surfaces and exogenous activators, others are modified on platelet activation and act as receptors. These glycoproteins are, therefore, of special importance to the haemostatic function of blood platelets (McEver, 1990).

Platelets are formed by the fragmentation of their precursor cells, megakaryocytes, which arise from stem cells. Because of its large size and DNA content, each megakaryocyte is capable of producing approximately 2000 platelets (Mazur, 1987; Stenberg and Levin, 1989). At some point in their development, megakaryocytes enter a phase of cytoplasmic growth with acquisition of platelet proteins and organelles (Mazur, 1987; Ebbe, 1976). While the mechanism of platelet release is not completely understood, some researchers believe that prior to platelet production, the megakaryocytes acquire a network of channels and demarcation membranes which divide into platelet-sized domains (Radley and Haller, 1982; Radley, 1986). Platelets are believed to form in the lung when megakaryocytes or their fragments are released into venous circulation (Trowbridge et al., 1982). The nuclei remain behind in the bone marrow where they are destroyed by reticulo-endothelial cells (Radley and Haller, 1983).

It is believed that enough nucleotides are contained in each platelet to maintain

it for a lifetime of 8-11 days. As platelets age they undergo changes in size, density, metabolism and function. Younger platelets are usually more active and larger in size. As they age their activity steadily decreases and terminates in their removal by the reticulo-endothelial system (Frujmovic and Milton, 1982; Wong et al., 1989).

Section II. Platelet Responses

Circulating platelets have two main functions *in vivo*: 1) to arrest bleeding and 2) to maintain vascular integrity (Packham and Mustard, 1984). When certain foreign surfaces or specific agonists interact with receptors on platelets, they induce the platelet functional response which consists of shape change, adhesion, aggregation, and secretion of granule contents (Colman, 1991; Holmsen, 1989).

A. Platelet Shape Change

The response of platelets to certain stimuli involves a rapid shape change in which they loose their discoid shape to a more rounded form, extend pseudopods (Holme, 1986) and become adherent to exposed surfaces. Platelet shape change may begin less than 5 sec after exposure to stimuli and occurs independently of extracelluar calcium and fibrinogen (Holmsen, 1989). The formation of pseudopods is associated with altered membrane structure and glycoprotein rearrangement (Nurden, 1987), while microfilament formation provides a support structure for advancing pseudopods (Oster and Perelson, 1987).

B. Platelet Adhesion

Following blood vessel injury, platelets adhere to collagen, basement membrane, and the myofibrils surrounding elastin in the subendothelium (Balduini et al., 1987). At high shear rates, as in capillaries and smaller blood vessels, platelet adhesion becomes dependent on the binding of von Willebrand factor to a specific adhesion receptor on platelet membranes (Balduini et al., 1987; Weiss, 1991). This receptor consist of a complex of two platelet membrane glycoproteins, glycoprotein Ib and glycoprotein IX (Stel et al., 1985; McEver, 1990). The von Willebrand factor is believed to serve as a bridge between glycoprotein Ib and IIb-IIIa in platelets and vessel wall collagen (Weiss, 1991). Following adhesion, platelet secretion and thromboxane A_2 production takes place which aids in recruitment of other platelets into the platelet plug (Packham and Mustard, 1984).

C. Platelet Aggregation

Platelet aggregation is distinct from platelet adhesion in that it involves the attachment of platelets to other platelets rather than to physiological barriers or *in vitro* surfaces. This process, *in vivo*, results when newly activated platelets adhere to each other, as well as, to collagen fibril-bound platelets. Platelet-platelet binding occurs following the expression of fibrinogen receptors on the surface of activated platelets (Apitz-Castro et al., 1991). This is dependent on calcium mobilization within the platelet allowing an increase in the accessibility of glycoprotein IIb and IIIa

through the binding of these glycoproteins to calcium. The glycoprotein IIb-IIIa complex then forms the fibrinogen receptor on the platelet surface, and fibrinogen links the adjoining platelets (Beardsley, 1990). These fibrinogen-bound platelets attach to other platelets and form a platelet clump or a thrombus. Other proteins like fibronectin, thrombospondin, and von Willebrand factor also bind to the glycoprotein IIb-IIIa complex. The interaction of these proteins with the glycoprotein IIb-IIIa complex is believed to increase aggregation and the adherence of platelets to exposed endothelial surfaces (Balduini et al., 1987). Interplatelet bridging may also involve the binding of aggregated immunoglobulins to Fc receptors on the platelet surface (Packham and Mustard, 1984). Interplatelet bridging mechanisms may occur independently or together to insure aggregation *in vitro*, or *in vivo* at the site of injury (Balduini et al., 1987).

The measurement of platelet aggregation *in vitro* has played a major role in the development of our current understanding of platelet function. This is partly due to an assumed relationship between aggregation *in vitro* and platelet function *in vivo*(Born and Hume, 1967; Huang and Detwiler, 1986). Under physiological conditions, platelets are usually exposed to more than one aggregating agent which augment the aggregation response to a level that is greater than the sum of the individual responses.

Studies of platelet aggregation *In vitro* usually involve the use of a single platelet agonist and either platelet rich plasma (PRP) or platelets that are washed and

suspended in an appropriate buffer. Aggregation is observed by a photometric method using an aggregometer adjusted to detect changes in light transmission through a stirred suspension of platelets together with agonist. Any changes in light transmission are recorded as a function of time and can be compared for agonist effects (Zucker, 1980). Using this method, 80-90 % of all platelets aggregate before increased light transmission is observed. Therefore, what is measured are the events occurring later in the aggregation process, such as clumping together of platelet aggregates, which are largely dependent on earlier events (Jamaluddin and Krishnan, 1987). This method offers the advantages of convenience and simplicity.

Two patterns of aggregation can be distinguished *in vitro* depending on the strength of the aggregating stimuli (Balduini et al., 1987; Chario et al., 1977; Huang and Detwiler, 1986). Using a strong stimulus, such as thrombin, or high concentrations of other agonists, aggregation is a single phase event. However, with weak stimuli aggregation may occur in two phases. The first phase, known as primary aggregation, involves the binding of fibrinogen to platelets resulting in cell-cell linkages that are loose and reversible. Secondary aggregation is irreversible, possibly because of reinforcement of the fibrinogen cell-cell linkage with thrombospondin (Balduini et al., 1987). Complete irreversible aggregation can only be achieved during the secondary phase.

D. Platelet Secretion

Platelets have some features of secretory cells. They take up and store compounds, and then release them by exocytosis during a secretion reaction (Holmsen, 1987). During platelet secretion, platelets release the contents of their three classes of secretory granules into the extracelluar environment to different degrees depending on the strength of stimulation (Huang and Detwiler, 1986). Some of the secreted substances are themselves platelet agonists and enhance platelet stimulation by positive feedback. These substances act synergistically with the primary agonist to augment the overall platelet response (Schror and Braun, 1990).

Section III. Signal Transduction Mechanisms in Platelets

Platelet activation is dependent on the transferral of the surface signal by the activation of a number of signal transduction pathways. These pathways, therefore, mediate the morphological responses of platelets to stimuli.

One of the first studied mechanisms of signal transduction involved modulation of adenylate cyclase by certain stimuli through stimulatory and inhibitory G-proteins (Litosch and Fain, 1986; Tremblay and Hamet, 1987). When cAMP concentrations increase in platelets, protein kinase A is dissociated to yield its free catalytic subunit. This kinase in turn phosphorylates several distinct membrane and cytosolic proteins (Scrutton and Knight, 1987). Protein kinase A-mediated phosphorylation of the membrane proteins P22 or P24 results in activation of calcium pumps which promptly

remove calcium from the cytosol into the dense tubular system and close calcium channels. A similar but controversial role for cGMP has also been suggested (Tremblay and Hamet, 1987). Although most platelet agonists inhibit adenylate cyclase, the physiological significance of this inhibition is unknown. Adenylate cyclase activity is increased by agents such as prostaglandin I_2 or adenosine (Haslam et al., 1978a and b; Best et al., 1977), which inhibit platelet aggregation.

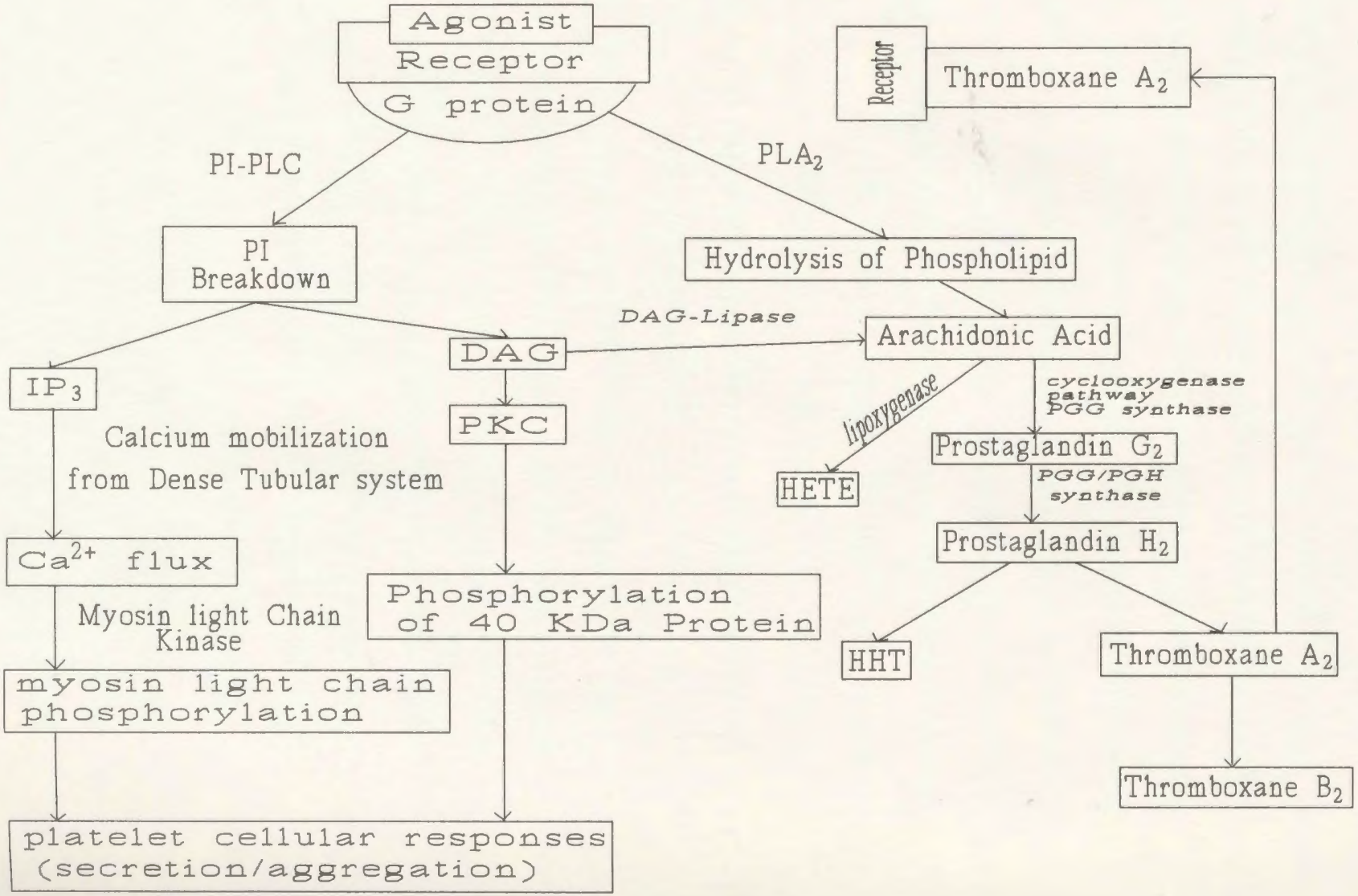
Calcium is a key signalling substance which is mobilized on platelet activation. Calcium can enter the platelet cytosol via receptor-mediated gating in the plasma membranes in addition to that released from internal stores in response to inositol-1,4,5-triphosphate (IP_3) (Pollock et al., 1987; Merritt and Hallam, 1988; Magosci et al, 1989). The nature of these channels, however, is virtually unknown.

Recently, attention has focused on the role played by hydrolysis of phospholipid in signal transduction mechanisms. This has lead to extensive study of the role of two enzymes, phosphatidylinositide-specific phospholipase C (PI-PLC) and phospholipase A_2 (PLA_2) in platelet activation (Fig. 2). These enzymes hydrolyse platelet membrane phospholipid, to generate two important second messengers, viz, 1,2-diacylglycerol (DAG) and IP_3, and also arachidonic acid which is subsequently metabolized to the potent but highly unstable interplatelet mediator, thromboxane A_2.

A. Phospholipase A_2 (PLA_2)

Activation of platelets with certain agonists results in a cascade of events

Fig. 2. **Role of PLA_2 and PI-PLC in platelet function.** Pathways involved in the metabolism of platelet phospholipid when PI-PLC and PLA_2 are activated as a result of the interaction of an agonist with its receptor.


Agonist
Receptor
G protein
PI-PLC
PLA2
PI
Breakdown
Hydrolysis of Phospholipid
DAG-Lipase
DAG
Arachidonic Acid
IP3
PKC
lipoxygenase
cyclooxygenase
pathway
PGG synthase
Calcium mobilization
from Dense Tubular system
HETE
Prostaglandin G2
PGG/PGH
synthase
Ca2+ flux
Prostaglandin H2
Phosphorylation
of 40 KDa Protein
Myosin light Chain
Kinase
HHT
Thromboxane A2
myosin light chain
phosphorylation
Thromboxane B2
platelet cellular responses
(secretion/aggregation)
Receptor
Thromboxane A2

12a

eventually leading to activation of PLA_2 (Billah et al, 1980; Billah et al., 1981). The action of this enzyme on membrane phospholipids result in hydrolysis at the *sn*-2 position leading to a preferential release of arachidonic acid (Irvine, 1982) which may be converted to active metabolites like prostaglandins, thromboxanes (Arita et al., 1989), and leukotrienes by the cyclo-oxygenase and lipoxygenase pathways (Siess et al., 1983). In addition, the hydrolysis of 1-O-alkyl-phosphatidylcholine by PLA_2 results in generation of 1-O-alkyl-2-lyso-*sn*-glyceryl-3-phosphocholine (lyso-PAF) which may be subsequently converted to 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (platelet-activating factor; PAF), a potent inflammatory mediator (Koltai et al, 1991a and b). PLA_2 activity appears to be quantitatively most important in the generation of arachidonic acid following platelet activation. The activity of DAG-lipase on arachidonic acid-rich DAG, formed following activation of PI-PLC (Bell et al., 1979; Rittenhouse, 1983) is believed to be only a minor contributor to arachidonic acid formation (Broekman, 1986).

When arachidonic acid is added exogenously to human platelets or released as a result of agonist-induced stimulation, it is metabolized by 2 oxygenase enzyme systems (Needleman et al., 1986). The cycloxygenase/thromboxane synthetase pathway synthesises prostaglandin endoperoxides (prostaglandin G_2 and prostaglandin H_2) and thromboxane A_2 (Hamberg et al., 1975) which induce platelet aggregation and secretion (Hamberg et al., 1974). Cycloxygenase activity also catalyses the formation of 12-hydroxyheptadecatrienoic acid (HHT) and malonyl dialdehyde

(Lagarde, 1988). The lipoxygenase pathway converts arachidonic acid into 12-hydroperoxyeicosatetraenoic acid, which is further reduced by the associated peroxidase into 12-hydroxyeicosatetraenoic acid (HETE), believed to inhibit platelet aggregation by competing for the prostaglandin H_2/thromboxane A_2 receptor (Croset and Legarde, 1983).

Thromboxane A_2 is the most important metabolite of arachidonic acid in platelets. Since its discovery in 1975 (Hamberg et al., 1975), activation mechanisms of thromboxane A_2 in platelets have been extensively studied (Arita et al., 1989). The biological half life of thromboxane A_2 is very short and it is quickly converted to its stable metabolite, thromboxane B_2. Recent studies suggests that all its effects are mediated by specific receptor proteins present on the platelet membrane (Halushka et al., 1987). Studies using thromboxane A_2-like agonist, U46619, reveal two classes of binding sites in human platelets, a high affinity binding site which mediates shape change and a low affinity binding site which results in aggregation and secretion reactions (Arita et al., 1989). Surprisingly, rat platelets contain only one class of binding sites which provoke only platelet shape change. For this reason, thromboxane A_2 can be classified as a partial agonist in rat platelets (Arita et al., 1989).

The mechanisms of signal transduction leading to arachidonic acid mobilization by PLA_2 are not well understood. There is, however, considerable evidence to suggest involvement of a specific G-protein (Greco and Jamieson, 1991). This G-

protein is believed to be pertussis toxin-sensitive, associated with ADP-ribosylation of a 41 kDa membrane protein, and is independent of the G-protein involved in the hydrolysis of phosphatidylinositides (PI) by PI-PLC (Nakashima et al., 1987). A number of other factors such as the Na^+-H^+ exchanger (Sweatt et al., 1986), lipocortin (Touqui et al., 1986), calcium (Baron and Limbird, 1988), protein kinase C (PKC) (Banga et al., 1991) and cGMP (Sane et al., 1989) have been implicated in the modulation of PLA_2 activity.

B. Phosphatidylinositide-Specific Phospholipase C (PI-PLC)

Binding of a wide variety of agonist to their cell surface receptors results in increased PI turnover (Michell, 1975). The primary response to ligand-receptor interactions leads to activation of a PI-PLC which cleaves PIs, including phosphatidylinositol-4,5-bisphosphate (PIP_2), and generates two second messengers, IP_3 and DAG (Berridge, 1987). PI-PLC may also hydrolyse phosphatidylinositol and phosphatidylinositol-4-monophosphate which leads to generation of DAG but not IP_3 (Nishizuka, 1989a). However, the relative importance of the different PIs as substrates for PI-PLC remains unclear (Tysnes et al., 1991). PI-PLC is widely distributed in many mammalian tissues and is found as either a membrane bound form, sometimes linked to G-proteins (Carter et al., 1990), or as a cytosolic form. Purification and molecular cloning of these enzymes have revealed several families of PI-PLC (Rhee et al., 1989; Kritz et al., 1990; Meldrum et al, 1991).

Although little is known about the biochemistry of the events occurring between the receptors and PI-PLC activation, G-proteins are believed to mediate this interaction (Litosch and Fain, 1986; Hrbolich et al., 1987). Preceding platelet activation, a stimulatory agonist binds to its platelet membrane receptor, activates a G-protein which in turn activates PI-PLC (Fig. 2). The IP_3 produced stimulates the release of calcium from internal stores (O'Rourke et al., 1985). DAG that remains in the membrane is involved in the activation of the calcium-activated phosphatidylserine (PS)-dependent PKC (Nishizuka, 1984). In the presence of released calcium, DAG converts inactive PKC into an active form, possibly by a covalent modification of the enzyme within or near the catalytic domain (Pelech et al., 1990). Increasing evidence also points to a critical role for DAG in the development of membraneous intermediates that promote membrane fusion during exocytosis (Das and Rand, 1984; Siegel et al., 1989).

Stimulation of PKC by a number of agonists has been shown to result in the phosphorylation of a 40-47 kDa platelet protein (Watson et al., 1988; Yamada et al., 1987; Krishnamurthi et al., 1989) which is thought to aid in granule secretion by promoting association between the open canalicular system and the granule membranes (Gerrard et al., 1985). This process is also aided by granule centralization, mediated by IP_3-induced release of calcium from intracellular stores (Isreals et al., 1985; Brass et al., 1987). Increased cytosolic calcium activates a calcium/calmodulin-dependent myosin light-chain kinase that phosphorylates myosin

light-chains. This is a prerequisite for the actin activation of the Mg^{2+}-ATPase activity and, therefore, contraction of the actin-myosin cytoskeleton (Dabrowska and Hartshorne, 1978; Hathaway and Adelstein, 1979) and centralization of granules, which plays an important role in platelet shape change, aggregation and secretion (Nishakawa et al., 1980; Daniel et al., 1984). Therefore, IP_3 and DAG are thought to act in synergy to produce secretion and aggregation responses in the platelets (Kaibuchi et al., 1983).

Both IP_3 and DAG rapidly formed by PI-PLC are also rapidly removed by various conversions in which their chemical moieties rejoin the PI pool. Thus, the formation of the signal molecules are part of a cyclic process (Holmsen, 1989) which maintains steady state levels of phosphatidylinositol, phosphatidylinositol-4-phosphate, and PIP_2. The transient accumulation of DAG following PI-PLC stimulation is quickly followed by phosphorylation of DAG to phosphatidic acid (PA) by DAG kinase and/or hydrolysis by DAG lipase (Rittenhouse-Simmons, 1979; Bell et al., 1979; Billah et al., 1979). PA, is also a potential second messenger (Putney et al., 1980; Imai et al., 1982) causing calcium influx across the plasma membrane in intact cells.

Section IV. Role of Agonists in Platelet Activation

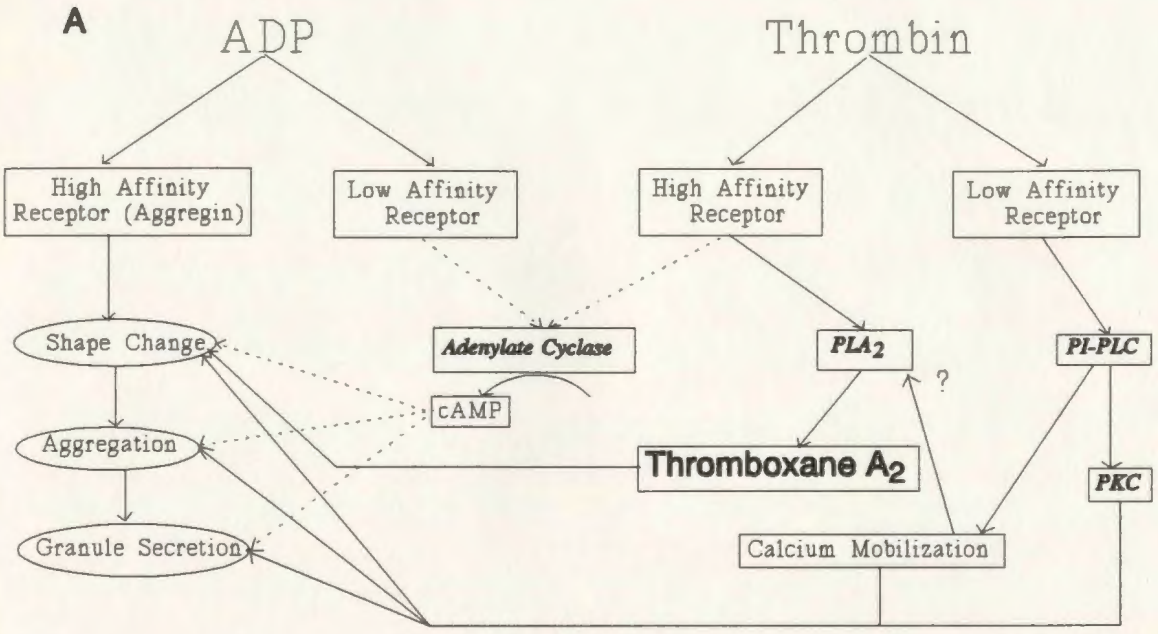
Platelet agonists can be partially categorized as being either weak or strong (Charo et al., 1977). Weak agonists include ADP, PAF, and epinephrine, while

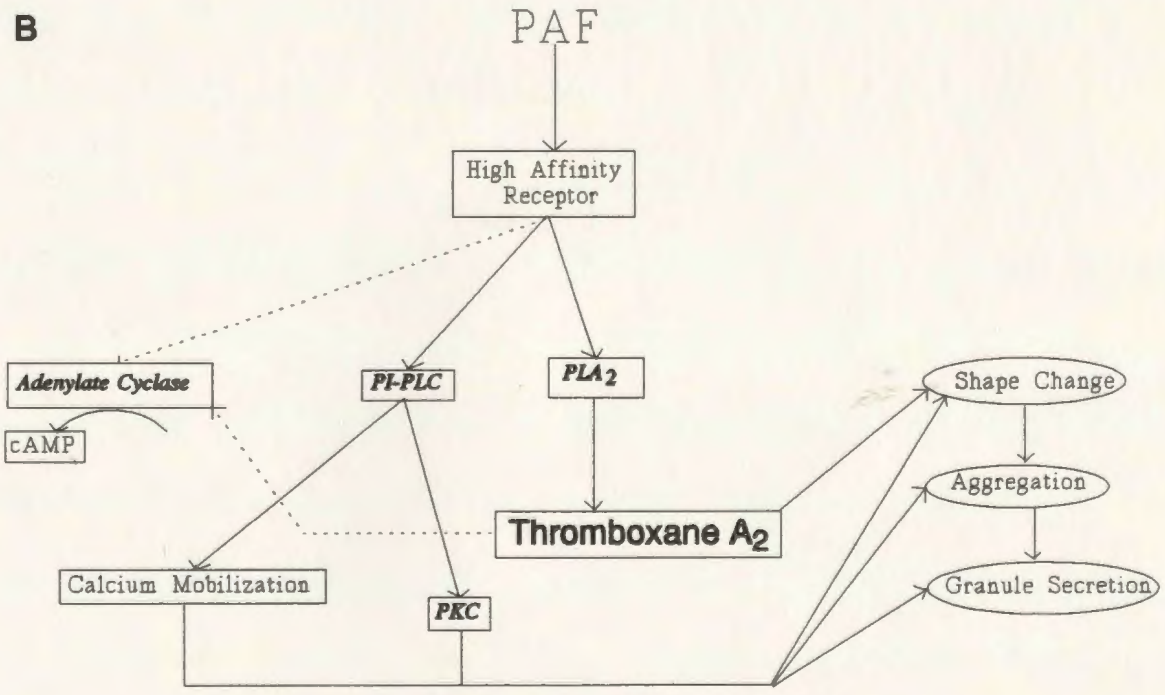
thrombin, collagen and A23187 (a calcium ionophore) are examples of strong agonists. Low concentrations of strong agonists behave in a fashion similar to that of a weak agonist (Huang and Detwiler, 1986). *In vitro* the activation by weak agonists may, however, more accurately reflect the regulatory mechanisms that are important in platelet physiological function (Weiss and Aledort, 1967; Huang and Detwiler, 1986). Weak agonists will only trigger platelet shape change and aggregation, and cause secretion of granule contents through positive feedback mechanisms (Holmsen, 1989). Strong agonists trigger all three platelet responses independently (shape change, aggregation and secretion) *in vitro*.

A. Adenosine Diphosphate (ADP)

ADP is not only released from injured endothelial or red cells, but is also released from platelet dense granules. ADP has been known to induce a number of responses in platelets through interaction with high and low affinity binding sites on the platelets (Fig. 3A) (Coleman, 1990). This is followed by a number of events including the formation of a complex between glycoprotein IIb-IIIa and calcium, centralization of internal granules, mobilization of internal calcium, phosphorylation of several proteins, and inhibition of prostanoid-induced adenylate cyclase activity (Born, 1962; Mills, 1982; Hawiger et al., 1987). The binding sites responsible for calcium mobilization and inhibition of adenylate cyclase are distinct from those responsible for shape change and aggregation, that is, the ADP-receptor, aggregin

Fig. 3. **Role of ADP, thrombin and PAF in platelet activation.** General schemes for the involvement of receptors and signal transduction pathways in the response of platelets to A) ADP and thrombin, and B) PAF. Solid lines (——) indicate stimulatory actions while dotted lines (·····) indicate inhibitory actions (Adapted from Coleman, 1990; Greco and Jamieson, 1991; Braquet et al., 1987).


A
ADP
Thrombin
High Affinity Receptor (Aggregin)
Low Affinity Receptor
High Affinity Receptor
Low Affinity Receptor
Shape Change
Adenylate Cyclase
PLA2
PI-PLC
cAMP
?
Aggregation
Thromboxane A2
PKC
Granule Secretion
Calcium Mobilization


B
PAF
High Affinity Receptor
Adenylate Cyclase
cAMP
PI-PLC
PLA2
Shape Change
Aggregation
Thromboxane A2
Calcium Mobilization
PKC
Granule Secretion

(Fig. 3A) (Mills, 1985; Rao and Kowalska, 1987; Colman et al., 1990). The role of PI-PLC in ADP-mediated platelet activation is controversial. Some studies have shown that ADP-induced platelet activation does not involve PI hydrolysis, since in the presence of indomethacin, neither A23187 (Rittenhouse, 1984) nor ADP (Fisher et al., 1985) could activate platelets through PI hydrolysis. Other reports have indicated an initial rapidly reversible phosphorylation of the 20 and 47 kDa proteins (Daniel et al., 1984; Carty and Gear, 1985), a process believed to be mediated by DAG-activated PKC activity. In the presence of fibrinogen, and suppressed thromboxane A_2 formation, one gets only primary aggregation and shape change without secretion (Rink and Hallam, 1984). Even though only modest amounts of thromboxane A_2 are produced following stimulation with ADP, these amounts appear to be sufficient to stimulate a secretory response in most species.

B. Platelet-Activating Factor (PAF)

PAF was first discovered in the early 1970's by Benveniste et al. (1972) as a compound present in the plasma of rabbits undergoing anaphylaxis. The structure of the lipid factor was later determined to be 1-O-alkyl-2-acetyl-*sn*-glycerol-3-phosphocholine (Benveniste et al., 1979; Demopoulos et al., 1979; Blank et al., 1979). PAF is not only the most potent platelet aggregating agent known, but it is also highly active in several other systems and has been shown to be an important component in inflammatory and allergic responses (Braquet et al., 1987). PAF is

formed and released by immunoglobulin E-sensitized basophils, stimulated macrophages or neutrophils, or by platelets themselves following activation.

The amount of PAF present in biological fluids is regulated by a number of factors. These include the amount of PAF synthesised within the cell following stimulation, the amount of PAF that is actually released, and the rate of catabolism of PAF into inactive products (Snyder, 1985). PAF biosynthesis occurs predominantly through the activities of two membrane associated enzymes, PLA_2 and acetyltransferase acting sequentially on 1-O-alkyl-2-acyl-glycerol-3-phosphocholine.

The catabolism of PAF depends on the enzyme acetylhydrolase which degrades PAF to the inactive lyso-PAF (Braquet et al., 1987). This enzyme is localized in both the cytosol and in plasma. The plasma PAF-acetylhydrolase was first described by Farr et al. (1983) as a plasma acid-labile factor associated with lipoproteins (Farr et al., 1983; Stafforini et al., 1987). This extracelluar PAF-acetylhydrolase plays a central role in regulating extracelluar concentrations of PAF.

At concentrations ranging from 10^{-8}-10^{-11} M, PAF causes platelet shape change, release of granule contents and thromboxane A_2, and aggregation of platelets (Braquet et al., 1987). The sensitivity of platelets to aggregation by PAF varies greatly from species to species, with the guinea pig being the most sensitive, followed by rabbit, which is one or two orders less sensitive, depending on the platelet suspension medium. Human, baboon, and canine platelets are even less sensitive to PAF (Braquet et al., 1987). The platelets of rats are refractory to PAF-induced

aggregation (Sanchez-Crespo et al., 1981) and high affinity receptors for PAF have not been found in rat platelets (Inarrea et al., 1984).

The first step in activation of platelets by PAF is believed to involve interaction of PAF with its receptor located on the platelet membrane (Fig. 3B). These receptors are of high affinity and low capacity, numbering between 42 and 20,000 per platelet (Hwang, 1990). The binding of PAF to this receptor usually results in PI breakdown (Morrison and Shukla, 1988), mobilization of calcium (Sage and Rink, 1987), hydrolysis of GTP (Hwang et al., 1986), phosphorylation of 20 kDa and 40 kDa proteins by PKC (Sugatani and Hanahan, 1986) and release of arachidonic acid, followed by platelet aggregation and secretion (Hwang et al., 1983).

C. Thrombin

Thrombin is one of the most effective platelet stimuli. It can induce shape change, secretion, and full aggregation at concentrations as low as 0.04 U/ml (Lapetina, 1990; Greco and Jamieson, 1991). At low concentrations of thrombin, secretion from α-granules is required for aggregation (Harfenist et al., 1982). At high concentrations of thrombin platelet responses, such as aggregation and α- and dense-granule secretion, occur independently (Greco and Jamieson, 1991; Kunicki et al., 1983).

Thrombin stimulates platelet aggregation and secretion through two receptors of high and moderate affinity on the platelet surface (Fig. 3A). The high affinity

receptor is believed to be associated with G-protein-dependent activation of PLA_2 (Greco and Jamieson, 1991) and inhibition of basal adenylate cyclase. Thrombin is very active in releasing arachidonic acid from platelets by PLA_2 stimulation (Lapetina, 1982 and 1990), making thrombin the most effective stimulus for thromboxane A_2 formation. The moderate affinity receptor, believed to be responsible for PI-PLC and PKC activation, may be linked to substrate proteolysis by thrombin. The activation of PI-PLC by thrombin appears to lead to preferential hydrolysis of PIP_2 (Lapetina, 1986), but phosphatidylinositol and phosphatidylinositol-5-monophosphate are also hydrolysed and serve as a source of DAG (Lapetina, 1990). Synergistic activation of both PLA_2 and PI-PLC pathways are required for maximum activation of human platelets.

D. Other Agonists

The adherence of collagen to platelets is believed to provide the first step towards the formation of a haemostatic plug or a thrombus on the subendothelium. In fact, fibrillar collagen is considered the most thrombogenic agent of the vascular endothelium (Parmentier et al., 1990). The resultant platelet aggregation, which follows the adhesion of platelets to collagen, is believed to result from the presence of ADP and thromboxane A_2 which are released from platelets. Therefore, removing ADP or inhibiting thromboxane A_2 formation results in strong inhibition of collagen-induced aggregation (Packham and Mustard, 1984).

The divalent cation ionophore, A23187 has been used extensively in the study of platelet responses to increased intracellular calcium. This agonist has been shown to cause platelet aggregation, secretion, and activation of the arachidonate pathway (Packham and Mustard, 1984).

Other platelet activating substances acting through specific receptors are epinephrine, norepinephrine, and serotonin. Epinephrine and norepinephrine interact with the α_2-adrenergic receptors on the platelet surface and cause platelet aggregation along with inhibition of adenylate cyclase (Colman, 1990; Packham and Mustard, 1984)). Platelets also have receptors for serotonin on their surface that are responsible for its weak aggregating effects (Baumgarter and Born, 1968). By itself serotonin can induce aggregation at unphysiologically high concentrations (Baumgarter and Born, 1968; Packham and Mustard, 1984). It is likely that the physiological role of these neuro-transmitters involves their ability to potentiate aggregation induced by low concentrations of other aggregating agents (De Clerck, 1990; Baumgarter and Born, 1968; Packham and Mustard, 1984).

The platelets of certain species contain Fc receptors. (Packham and Mustard, 1984). The binding of immunocomplexes to these receptors results in a sequence of reactions which include shape change, aggregation and secretion. The bridging of certain surface molecules with immunoglobulins may provide the signal for platelet activation (Henson and Ginsberg, 1981).

CHAPTER 2

PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C (PC-PLC) AND D (PLD)

In chapter 1 the roles of PLA_2 and PI-PLC in platelet function were described. Since a definitive role for PC-PLC and PLD in platelet function has not been reported, the role of these enzymes in mediating cellular reactions in other tissues are discussed separately in this chapter.

Section I. Background and Cellular Origins

Phosphodiesteric cleavage of phosphatidylcholine (PC) has been described in a number of different tissues. This hydrolysis of PC to form either phosphorylcholine and DAG, or choline and PA is due to the action of either phosphatidylcholine-specific phospholipase C (PC-PLC) or phospholipase D (PLD), respectively. Although, PC-PLC or PLD in different tissues may carry out similar reactions, their substrate specificity and requirements for activity may differ.

A number of cell-free preparations have been shown to degrade PC by PC-PLC. Several criteria have been used to characterize these PC-PLCs including, pH optima, substrate specificity, detergent requirements, divalent cation requirements, subcellular localization and molecular size. For example, phospholipase C (PLC) of

lysosomal origin requires an acid pH for maximum activity, and it hydrolyses PE in addition to PC (Matsuzawa and Hostetler, 1980). PC-PLC from brain cytosol (Edgar and Freysz, 1982) and liver membranes (Irving and Exton, 1987) require alkaline pH for maximum activity. Cell-free preparations from rat endothelial cells also degrade exogenous PC by PC-PLC (Clark et al., 1986a; Martin et al., 1987). In contrast, PC-PLC from dog heart cytosol (Wolf and Gross, 1985), bull seminal plasma (Sheikhnejad and Srivastava, 1986), and promonocytic U937 cells (Clark et al., 1986b) exhibit neutral pH optima and utilize exogenous PC but not PI.

PLD was first discovered in plants (Hanahan and Chaikoff, 1947), and was long thought to be absent from human tissue. In 1975 a mammalian form of PLD with an acid pH optimum was identified by Saito and Kanfer (1975) in solubilized brain membrane preparations. Subsequent studies demonstrated PC-preferring PLD activity in homogenates and membranes from a number of other tissues and cells. These include lung, liver, adipose tissue, endothelial cells, HL-60 cells and spermatozoa (reviewed in Billah and Anthes, 1990). PLD activities have been partially purified from rat brain (Taki and Kanfer, 1979), lung (Kater et al., 1976), and human endothelial cells (Martin, 1988) and found to be associated primarily with the particulate fractions. A cytosolic PLD has also been described in several bovine tissues (Wang et al., 1991).

Section II. Role of PC-PLC and PLD in Signal Transduction Mechanisms

While the importance of PI as a source of signalling second messengers is now well established, it has become clear that PC can also generate lipid second messengers for signalling pathways (Billah and Anthes, 1990; Exton, 1990).

The generation of PC-derived DAG is believed to occur by either an indirect or direct pathway (Huang and Cabot, 1990). The direct pathway is catalysed by PC-PLC leading to generation of DAG. Operation of the indirect pathway results in generation of DAG by the sequential actions of PLD and PA phosphohydrolase.

Receptor-linked activation of PC-PLC and PLD may occur via several distinct mechanisms involving multiple factors (Fig. 1). Recent studies have indicated that many of the agonists that stimulate PIP_2 hydrolysis also stimulate PC-PLC and PLD which catalyse the breakdown of PC to form DAG, PA, choline and phosphorylcholine (reviewed by Billah and Anthes, 1990; Exton, 1990). This has presented the possibility that PI-PLC mediated increases in DAG and calcium levels may be a prerequisite for agonist-induced PC breakdown. This hypothesis is supported by studies showing that phorbol ester, an activator of PKC, stimulates PC hydrolysis leading to accumulation of PC-derived DAG and PA. DAG, a product of PI hydrolysis, and phorbol ester also act synergistically with A23187 to activate neutrophil PLD (Billah et al., 1989a). On the other hand, in studies using certain cultured cells, phosphodiesteric cleavage of PC was shown either to precede PI-

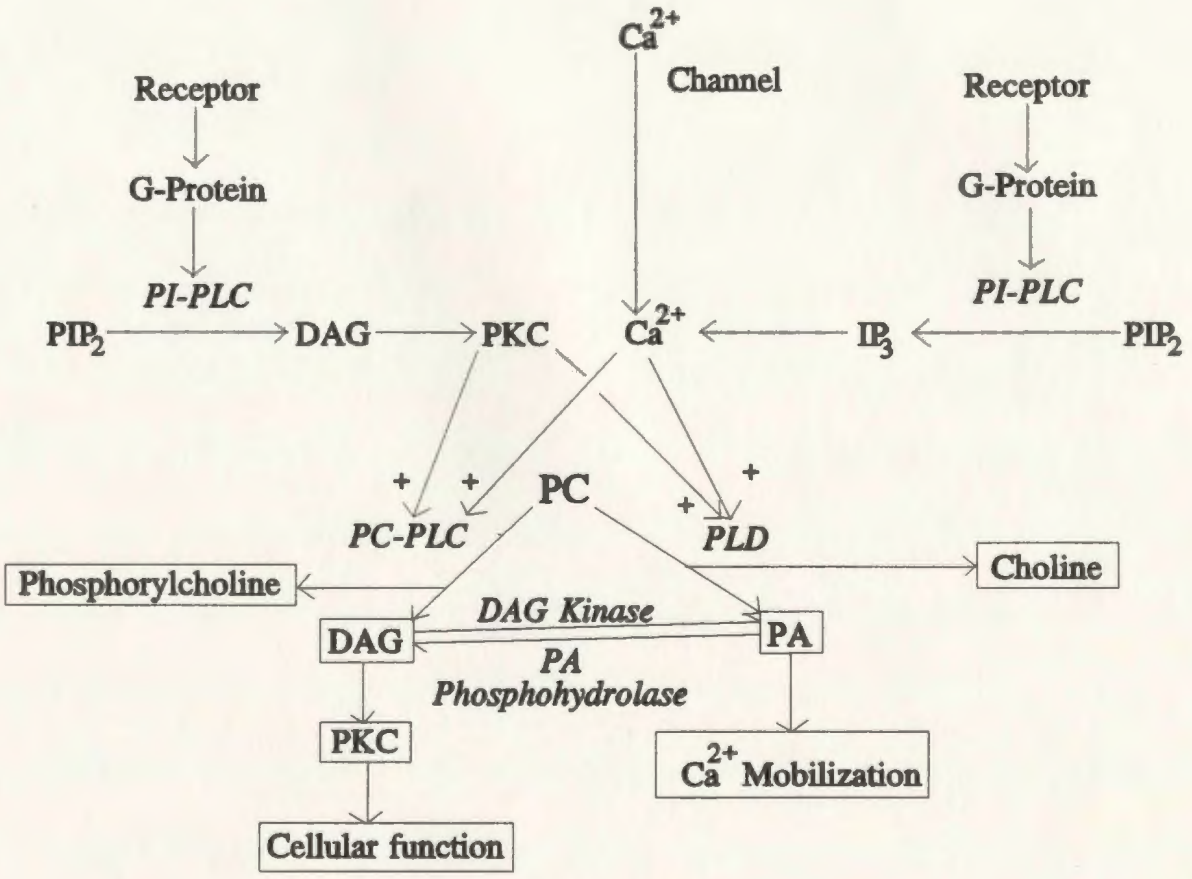


Fig. 1. **Role of phosphodiesteric cleavage of PC in signal transduction.** General scheme showing the involvement of receptors, calcium and/or G-proteins in the stimulation of PC-PLC or PLD required in signal transduction pathways (Adapted from Billah and Anthes, 1990; Exton, 1990).

hydrolysis (Slivka et al., 1988), or occur independently of PI hydrolysis (Duronio et al., 1989; Rosoff et al., 1988; Wright et al, 1988). These results indicate that although PI hydrolysis may be involved, it is neither essential nor sufficient for receptor-mediated PC breakdown.

A number of studies have demonstrated the role of calcium in PC hydrolysis by PC-PLC or PLD. In particular, neutrophil PLD activity stimulated by either receptor agonists or A23187 can be inhibited by depletion of extracelluar calcium using EGTA (Billah et al., 1989a and b; Cockcroft, 1984; Pai et al., 1988). In spermatozoa, agonist-induced calcium influx and stimulation of PLD are inhibited by the calcium channel blocker verapamil (Domino et al., 1989). In cell-free preparations, PC-PLC from rat brain (Taki and Kanfer, 1979), granulocyte homogenates (Anthes et al., 1989), heart (Wolf and Gross; 1985), and promonocytic U937 cells (Clark et al., 1986b) require calcium for maximum activity. In contrast, PLD activity in hepatocyte membranes (Bocckino et al., 1987), endothelial cell homogenates (Martin, 1988) and spermatozoal extracts (Tettenborn and Mueller, 1988) does not require calcium. It, therefore, appears that the requirement of exogenous calcium for PC hydrolysis may vary with tissue type.

The involvement of PKC as a prerequisite for PC-PLC activity varies from tissue to tissue. PKC activators like phorbol esters or synthetic DAGs also activate PC hydrolysis by PC-PLC or PLD (Billah and Anthes, 1990). However, inhibition or down regulation of PKC blocked phorbol ester-induced PC-PLC or PLD activity

partially in some tissues, and completely in others. While there is no evidence that PKC is the sole target for phorbol ester, it is possible that phorbol ester may interact directly with PLD and PC-PLC (Billah and Anthes, 1990; Kiss and Anderson, 1989). Since receptor-mediated PC-PLC (Besterman et al., 1986; Slivka et al., 1988) and PLD (Billah et al., 1989b; Liscovitch and Amsterdam, 1989) activities are not inhibited by PKC inhibitors, their activities may be governed by mechanisms independent of enhanced protein phosphorylation.

Emerging evidence suggests that G-proteins control PC hydrolysis by both PC-PLC and PLD. GTPγS, a G-protein activator, has been shown to activate PLD in hepatocyte membranes (Bocckino et al., 1987), granulocyte homogenates (Anthes et al., 1989), permeabilized endothelial cells (Martin and Michaelis, 1989) and PC-PLC in hepatocyte membranes (Irving and Exton, 1987; Bocckino et al., 1987). These effects could be inhibited by GTPßS, a G-protein antagonist. The effect of pertussis toxin is less clear, since in some tissues, pertussis toxin inhibits PC hydrolysis, while, in other tissues there is no effect. This indicates that PC-specific phosphodiesterases are regulated by distinct G-proteins that may, in some cases, be insensitive to pertussis toxin.

Based on these findings several models have been derived to explain the mechanism of receptor-mediated PC hydrolysis by PLC or PLD (Fig. 1). One mechanism may involve the activation of PI-PLC which hydrolyses PIP_2 to DAG and may activate PC-PLC or PLD by PKC. PC hydrolysis in some cells may be partially

due to the rise in cytosolic calcium by G-protein linked calcium channels or as a result of PI hydrolysis and subsequent calcium mobilization (Billah and Anthes, 1990).

The physiological significance of agonist-stimulated PC breakdown largely involves the important role of DAG in signal transduction pathways. It is believed that PC-breakdown could generate DAG for prolonged periods of time that would be required for sustained activation of PKC, since in most cells, the physiological response may persist long after the calcium signal returns to normal and the IP_3 signal has weakened (Billah and Anthes, 1990; Exton, 1990). In addition, since DAG species containing various fatty acids are able to activate PKC, it is also possible that different DAG subspecies affect different PKC isoforms to produce distinct PKC activation patterns (Billah and Anthes, 1990; Nishizuka, 1986; Nishizuka, 1989b).

CHAPTER 3

C-REACTIVE PROTEINS (CRP)

Section I. Introduction

A. Historical Background

In 1930, Tillet and Francis discovered a substance, in the sera of acutely ill patients, that precipitated the pneumococcal C-polysaccharide (CPS). This factor was called C-precipitin or C-reactive substance. In 1941, Abernethy and Avery further characterized human CRP as a protein and designated it as "C-reactive protein". The calcium-dependent interaction of human CRP with CPS was later used to isolate CRP in a crystalline form from a lipoprotein-rich fraction of human serum by a process that involved precipitating with CPS (McCarty, 1947; Wood et al., 1954). Volanakis and Kaplan (1971) demonstrated that the presence of a phosphorylcholine moiety on CPS (Fig. 1) was a possible binding site for CRP. Subsequently, Jennings et al. (1980) demonstrated the attachment of a phosphorylcholine moiety to the CPS.

B. Distribution of CRP

Since its original discovery, analogues of human CRP have been found in a number of other species. Anderson and McCarty (1951) first reported the presence of CRP in the serum of rabbits during the acute phase response. Subsequently, CRP was

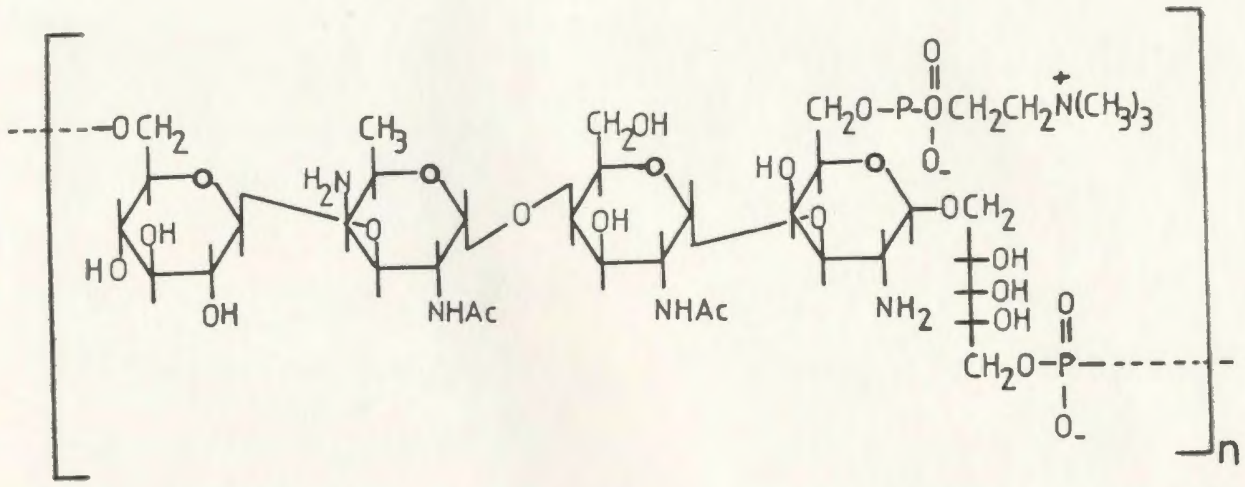


Fig. 1. **Structure of the CPS from *Streptococcus pneumoniae* Type 1.** Jennings et al. (1980) reported that CPS is a repeating unit composed of ß-D-Glu*p*-1→3-α-AAT-Gal*p*-1→4-α-D-GalNAc*p*-1→3-ß-D-GalNH_2*p*-1→1'ribitol-5-phosphate, where phosphocholine substituents, believed to be the primary binding site for human CRP (Volanakis and Kaplan, 1971), are situated at O(6) of the unacetylated galactosamine residues (AATGal is 2-acetamido-4-amino-2,4,6-trideoxygalactose).

found to be present in the sera of various species ranging from the invertebrates to the mammals. CRP has been found in lumpsucker (Fletcher et al., 1981), plaice (Pepys et al., 1982), rainbow trout (Winkelhake and Chang, 1982), dog fish (Robey et al., 1983), horseshoe crab (Robey and Liu, 1981; Fernandez-Moran et al., 1968), dog (Riley and Coleman, 1970), goat (Maudsley et al., 1987a), cow (Maudsley et al., 1987b), rat (Nagpurkar and Mookerjea, 1981), female syrian hamster (Coe, 1977) and monkey (Riley and Coleman, 1970). One important feature of CRP that has been conserved within most of these species is the calcium-sensitive property to bind to the phosphorylcholine ligand.

Section II. Human CRP

A. Biosynthesis

Since its discovery, human CRP has been described as a marker in a number of disease states involving inflammation and tissue damage. For this reason human CRP has been considered one of the acute phase proteins, increasing in concentration more than 1000-fold on inflammation or infection to levels of approximately 0.1-0.2 mg/ml of serum (Kilpatrick and Volanakis, 1991; Kushner, 1982). The induction of the CRP gene, located on the proximal arm of chromosome 1 (Floyd-Smith et al., 1986; Goldman et al., 1987), during inflammation has been reported to be under the control of both interleukin 6 and interleukin 1 in adult hepatocytes (Moshage et al., 1988) and human hepatoma cell lines (Goldman and Liu, 1987; Ganapathi et al.,

1988).

While the liver is believed to be the main site of CRP synthesis during acute phase response, a number of studies have suggested transcription and translation of CRP by a small number of peripheral blood lymphocytes (Ikuta et al., 1986; Kuta and Baum, 1986; Murphy et al., 1991). However, the relevance of this extrahepatic synthesis of CRP is not understood.

B. Structure

Among the different species that contain CRP, rabbit CRP appears to most closely resemble human CRP in molecular appearance, subunit composition and amino acid sequence (Bach et al., 1977; Pepys et al., 1978). When examined under an electron microscope, human and rabbit CRP appear as cyclic pentamers by the arrangement of their five identical subunits, and are, therefore, sometimes classified into a super family of proteins known as pentraxins (Osmand et al., 1977; Pepys and Baltz, 1983). The subunits of human and rabbit CRP contain 206 amino acids (Gotschlich and Edelman, 1965; Wang et al., 1982) and share significant amino acid sequence homology with each other (Gewurz et al., 1982; Bach et al., 1977). The protein is also non-glycosylated and the inter-subunit contacts are comprised of non-covalent forces. There is also evidence of an intra-disulphide bond within each subunit (Baltz et al., 1982). Like human CRP, rabbit CRP is also an acute phase reactant.

C. Binding Properties

1. Binding to Phosphorylcholine

Equilibrium dialysis studies have shown human and rabbit CRP to bind to approximately 1 molecule of phosphorylcholine per subunit with K_d of about 10 μM (Anderson et al., 1978; Rassouli et al., 1992). The binding specificity of human CRP for phosphorylcholine has an absolute requirement for both the choline and phosphate moieties. Substitution of the phosphate moiety with choline derivatives of sulphonate or sulphate either decreases or abolishes binding to human CRP (Young and Williams, 1978). However, the high binding affinity for phosphorylcholine compared to other phosphate monoesters also indicates the specificity of CRP for the positively-charged tetra-methyl-ammonium groups. This suggests that the phosphorylcholine-binding site on CRP may recognise the zwitterionic nature of phosphorylcholine (Barnum et al., 1982).

CRP can bind to 1 or 2 molecules of calcium per subunit (Gotschlich and Edelman, 1965). Calcium is believed to mediate the binding of phosphorylcholine to CRP by inducing a conformational change in the CRP molecule (Gotschlich and Edelman, 1965). The binding of calcium to CRP is also believed to stabilize the CRP molecule and, thereby, making it more resistant to heat- and urea-induced denaturation (Potempa et al., 1981).

Similarity has been recognised between the phosphorylcholine-binding site of anti-phosphorylcholine myeloma protein (HOPC 8) and the phosphorylcholine-binding

site on human CRP (Barnum et al. 1982). The sequence Phe-Tyr-Met-Glu which participates in phosphorylcholine-binding by myeloma proteins (Kabat et al., 1976) is believed to be analogous to Phe39-Tyr40-Thr41-Glu42 involved in phosphorylcholine-binding by human CRP (Young and Williams, 1978). A more recent study has shown that human CRP displays the same idiotype as another anti-phosphorylcholine myeloma protein (TEPC-15) (Swanson et al. 1991).

2. Binding to Ether Lipids

Apart from phosphorylcholine and calcium, a number of other ligands have been shown to interact with human CRP. Of particular interest is the interaction of CRP with various ether lipids including glycerol-1-mono-palmitoyl and octadecyl-glyceryl ethers. Interaction with the analogous ester substituted glycerols does not occur (Riley et al., 1958).

3. Binding to Galactose-Containing Polysaccharides

A number of laboratories have reported the binding of human CRP to galactose-containing polysaccharides, including de-pyruvated pneumococcal type 4 capsular polysaccharide (Higginbotham et al., 1970), snail galactans (Uhlenbruck and Karduck, 1979), agarose (Volanakis and Narkates, 1981), and leishmania galactans (Pritchard et al., 1985). These interactions are calcium-sensitive and can be inhibited by phosphorylcholine. Although phosphate groups are involved in the interaction of

human CRP with Helix pomatia galactan (Soelter and Uhlenbruck, 1986), it is not clear whether phosphate groups or carbohydrates are involved in the binding of CRP to the other glycans (Kilpatrick and Volanakis, 1991).

4. Binding to Cationic Molecules

Human CRP has been shown to bind to a wide variety of cationic substances such as poly-L-lysine and poly-L-arginine polymers, lysine-rich and arginine-rich histones, myelin basic protein, and leucocyte cationic protein (Siegel et al., 1974,1975; Di Camelli et al., 1980; Potempa et al., 1981; Dougherty et al., 1991; Gewurz et al., 1982). In the absence of calcium, appropriate concentrations of CRP and cationic ligand form complexes which lead to aggregation and precipitation (Di Camelli et al., 1980). These events are inhibited by calcium in the absence of phosphorylcholine but stimulated by calcium in the presence of phosphorylcholine (Potempa et al., 1981). Therefore, the polycation-binding site on CRP is believed to be distinct from the phosphorylcholine-binding site, but may be in close proximity and under the regulation of both calcium and phosphorylcholine (Gewurz et al., 1982; Di Camelli et al., 1980; Dougherty et al., 1991).

D. Biological Properties

Despite the growing body of knowledge regarding the various ligand interactions and the physical properties of CRP, little is known of the actual *in vivo*

function of CRP. However, experimental evidence suggests that CRP is a multifunctional protein that can initiate and modulate a broad range of interactions.

1. Complement Activation

Human CRP has been shown to play a role in activation of the classical complement cascade by binding to CPS or other phosphorylcholine-containing ligands (Kaplan and Volanakis, 1974; Volanakis and Kaplan, 1974). Kaplan and Volanakis (1974) observed that the addition of CPS to CRP-containing acute phase sera resulted in depletion of the classical pathway components indicating involvement of CRP in complement activation. The involvement of C1q in this process was demonstrated by the requirement of human C1q in activation of the complement system in guinea pig serum (Volanakis and Kaplan, 1974). C1q was later shown to bind and agglutinate CRP-coated surfaces (Claus et al., 1977).

The binding of human CRP to ligands like polycations (Siegel et al., 1975), positively charged liposomes (Richards et al., 1979), PC:lysophosphatidylcholine (LPC) liposomes (Volanakis, 1982; Volanakis and Narkates, 1981) and nuclear DNA (Robey et al., 1985) have also been shown to activate complement. Complement activation involving these ligands resulted in opsonization and haemolysis (Mortensen et al., 1976; Osmand et al., 1975), which were inhibited by phosphorylcholine. These studies indicate a role for CRP, and its phosphorylcholine-binding site, in host defence by activation of the complement.

2. Binding to Damaged Cells and Model Membranes

It is believed that CRP does not bind to intact membranes of living cells but binds to cells with altered or damaged membranes (Volanakis and Wirtz, 1979; Volanakis and Narkates, 1981). *In vitro* studies of the binding of human CRP to PC-containing liposomes indicated that a disturbance of the molecular organization of the bilayer, by LPC, was necessary for binding to human CRP to liposomes (Volanakis and Wirtz, 1979). These studies provide a possible biochemical explanation for the binding of CRP to damaged membranes.

It is believed that CRP recognizes damaged cells *in situ*, and by activating the complement pathway (Volanakis and Wirtz, 1979), generates the chemotactic and opsonic activities required to promote phagocytosis. This leads to the eventual resolution and repair of the lesion (Volanakis, 1982).

3. Binding to Nuclear Contents

A number of nuclear contents including chromatin, histones, and small nuclear ribonuclear proteins have been shown to bind to human CRP (Du Clos, 1989; Du Clos et al., 1981, 1990, 1991; Robey et al., 1984, 1985). Human CRP has been shown to precipitate nucleosome core particles from chicken erythrocytes (Robey et al., 1984) and mediate the solubilization of chromatin by complement (Robey et al., 1985). In addition, human CRP was shown to bind to histone proteins in a manner that was calcium-sensitive and could be inhibited by phosphorylcholine (Du Clos et

al., 1991). It is believed that CRP may bind to chromatin that is released from dead cells during acute inflammation and mediate the removal of chromatin fragments from the body after cell death by complement-mediated phagocytosis (Robey et al., 1984).

4. Role in Opsonic Processes

It is generally believed that the function of CRP may be related to its specific recognition of foreign pathogens and damaged cells of the host and to initiate their elimination by interacting with humoral and cellular effector systems in the blood. The first opsonin-like property of CRP was demonstrated when human CRP was shown to induce agglutination and capsular swelling in certain types of *Streptococcus pneumoniae* (Lofstrom, 1944). Further support for a role of CRP as an opsonin came from studies which showed that certain bacterial species were more effectively phagocytosed in the presence of human CRP (Hokama et al., 1962). More recent studies have indicated that human CRP may bind, with certain components of the complement system, to cells and mediate phagocytosis by human monocytes (Mortensen et al., 1976). Other reports have shown the phagocytosis of CRP-opsonized cells is independent of the presence of complement (Kilpatrick and Volanakis, 1985; Kilpatrick et al., 1987). It is likely that during the opsonization process the phosphorylcholine-binding property of CRP may be involved in recognition and in the binding of a wide range of microorganisms or their degraded

products, by which their toxicity is reduced.

5. Interaction with Lipoproteins

Rabbit CRP in acute phase serum may exist as a complex with either low density lipoprotein (LDL) or very low density lipoproteins (VLDL) (Pontet et al., 1979; Cabana et al., 1982). The formation of these complexes between rabbit CRP and lipoproteins depend on the presence of apoprotein B in serum lipoproteins (Rowe et al., 1984a). Human CRP, on the other hand, does not bind to lipoproteins or form complexes with them *in vivo* (De Beer et al., 1982b). Nevertheless, in the serum of patients with type III hyperlipoproteinimia, which contains the abnormal lipoprotein, ß-VLDL, the formation of CRP-lipoprotein complexes was detected (Rowe et al., 1984b). This may indicate a role of CRP as a scavenger of abnormal lipoproteins.

It has also been suggested that the interaction between CRP and apoprotein B-containing lipoproteins may have functional relevance in cellular metabolism at the site of injury (Pepys et al., 1988). The binding of CRP to damaged cell membranes followed by interaction with apoprotein B-containing lipoproteins may result in specific localization of lipoprotein particles required for active cellular metabolism and processes of repair.

6. Effects on Phagocytic cells and lymphocytes.

Evidence for an interaction between CRP and immune cells was first described in 1937, by Abernethy and Francis, who observed a delayed skin reaction to CPS in

patients with elevated serum levels of CRP. It was later reported that moderate concentrations of purified human CRP, or acute phase sera with low levels of CRP, stimulated human polymorphonuclear leukocytes migration, whereas, high concentrations of human CRP inhibited this migration (Wood, 1951). Later studies showed a stimulatory role for CRP on inflammatory cell function, particularly when CRP was bound to particular ligands (Hokama et al., 1962; Ganrot and Kindmark, 1969; Kindmark, 1971; Mortensen et al., 1976).

Recent reports have provided a rather complicated picture of the role played by CRP in the regulation of various inflammatory cells (Kolb-Bachofen, 1991b). For example, the nature of the various effects of human CRP on neutrophils appear to differ depending on whether CRP is aggregated, cleaved or present in its native state. The opsonin-like modulatory influence of CRP is believed to stimulate neutrophil motility (Wood, 1951) and phagocytosis (Hokama et al., 1962; Ganrot and Kindmark, 1969; Kindmark, 1971), enhance neutrophil attachment to the endothelium (Muller and Fehr, 1986), increase monocyte chemotaxis (Whistler et al., 1986b), and increase tumourcidal activity (Barna et al., 1984). On the other hand, native CRP (< 10 μg/ml) can have a negative effect on cell function by inhibiting $O_2^{\cdot-}$ generation, chemotaxis, chemiluminescence, enzyme secretion, LPC-mediated lysis and protein phosphorylation in neutrophils (Buchta et al., 1987; Buchta et al., 1988; Tatsumi et al., 1988). Similarly, CRP hydrolysis products, formed by neutrophil-derived enzymes, also inhibit neutrophil function (Shephard et al., 1988). Likewise, the

interaction of CRP with lymphocytes has suggested a rather complicated role for CRP. Human CRP has been shown to inhibit rosette formation, proliferative responses to soluble antigens and to allogenetic cells (Mortensen et al., 1975), and inhibit the migration and formation of macrophage-chemotactic factor by stimulated lymphocytes (Mortensen et al., 1977). More recent studies have shown CRP to variously increase or decrease human B-lymphocyte colony formation (Whisler et al., 1983; Mackiewicz et al., 1985; Whisler et al., 1986a), and increase plaque formation (Mortensen et al., 1982). CRP also increased cell-mediated cytoxicity by T-lymphocytes (Vetter et al., 1986).

The effects of human CRP on inflammatory cells are believed to be mediated by specific binding sites on the cells. Specific binding sites for human CRP have been detected on neutrophils (Dobrinich and Spagnuolo, 1991), monocytes (Zeller et al., 1989; Ballou and Cleveland, 1991), macrophages (Zahedi et al., 1989), human promonocyte U-937 cells (Tebo and Mortenson, 1990), lymphocytes with natural killer activity (Ikuta et al., 1986; Kuta and Baum, 1986), antigen-stimulated lymphocytes (Croft et al., 1976) and peripheral blood lymphocytes (Oishi et al., 1973). Although, the calcium-dependent binding of human CRP to neutrophils (Dobrinich and Spagnuolo, 1991), and lymphocytes (Hornung, 1972) was inhibited by phosphorylcholine, the binding of human CRP to monocytes (Tebo and Mortenson, 1990) may be independent of the phosphorylcholine-binding site. A sub-population of lymphocytes with natural killer activity synthesise CRP and retain it on the surface

even in the presence of chelating agents (Ikuta et al., 1986; Kuta and Baum, 1986). Although a relationship between the CRP binding site on cells and the Fc receptor has been suggested, the nature of this relationship is not presently clear (Kilpatrick and Volanakis, 1991).

7. Effects on Platelets

Gewurz and Fiedel were first to demonstrate an inhibitory effect of human CRP on human platelet aggregation stimulated by ADP, epinephrine, collagen, poly-L-lysine, or thrombin (Fiedel and Gewurz, 1976a; Fiedel and Gewurz, 1976b; Fiedel et al., 1977). This effect did not involve calcium mobilization and was overcome by larger amounts of the agonists (Fiedel and Gewurz, 1976a). Their results suggested that the inhibitory effect of human CRP on platelet activities might be due to an interference with prostaglandin metabolism (Fiedel et al., 1977). Since a similar inhibitory effect was observed using anti-phosphorylcholine myeloma protein it was postulated that phosphorylcholine-binding proteins in general could modulate platelet reactivity by binding to phosphorylcholine moietys on the platelet surface (Fiedel et al., 1976). However, because of inconsistencies in the effects of different human CRP preparations on platelet function, it was later suggested that a low molecular weight factor of 8.3 - 12.5 kDa co-isolated with human CRP from pleural or ascitic fluids was responsible for the inhibitory properties of human CRP preparations (Fiedel et al., 1982a). These inhibitory properties were not observed when human CRP was

used in absence of the low molecular weight factor. Surprisingly, the inhibitory property of human CRP-low molecular weight, but not low molecular weight factor alone, was substantially reversed in the presence of CPS. More recently, it has been shown that cleaved forms of CRP, isolated from inflammatory fluids, as well as enzyme degraded forms of CRP also inhibit platelet activation (Fiedel and Gewurz, 1986). In contrast, Vigo (1985) reported that highly purified human CRP inhibited aggregation of rabbit platelets stimulated by thrombin or PAF and protected platelets against the lytic effects of LPC.

Studies using highly purified human CRP showed that aggregated or complexed CRP caused activation of platelets (Fiedel, 1988; Fiedel et al., 1982b-d; Fiedel, 1985; Fiedel, 1984). Thermally aggregated complexes of CRP, active oxygen-modified CRP (Miyagawa, 1988) and CRP-polycation complexes have been reported to activate or synergistically amplify platelet responsiveness similar to IgG (Fiedel, 1985). Furthermore, thermally aggregated CRP can cause platelets to undergo shape change, aggregation and secretion of α- and dense granule contents by a mechanism involving thromboxane A_2 production (Simpson et al., 1982). An uncharacterized receptor for aggregated CRP, distinct from the IgG Fc receptor has been proposed to mediate these responses (Fiedel et al., 1982b). Platelet aggregation induced by aggregated CRP is not inhibited by native CRP, indicating distinct receptors for aggregated and native CRP.

There have been a number of reports which suggest that highly purified human

and rabbit CRP inhibits PAF-induced aggregation of platelets (Vigo, 1985; Kilpatrick and Virella, 1985; Hokama et al., 1984; Filep et al., 1991). It was speculated that a CRP-PAF interaction played a role in the inhibitory effect of human and rabbit CRP on PAF-induced platelet aggregation. A recent study has also suggested that human CRP inhibited aggregation of human platelets by preventing the binding of PAF to its platelet membrane receptor (Filep et al., 1991).

Section III. Rat CRP

A. Isolation

During the course of a study on the effect of a serum protein factor in rat which inhibited the heparin-lipoprotein precipitation reaction (Mookerjea, 1978), a phosphorylcholine-binding protein was isolated and purified from rat serum by Nagpurkar and Mookerjea in 1981, using a sepharose-phenylphosphorylcholine affinity column. This protein was later shown to be identical to rat CRP independently isolated by Pontet et al. (1981) and De Beer et al. (1982a).

B. Structure

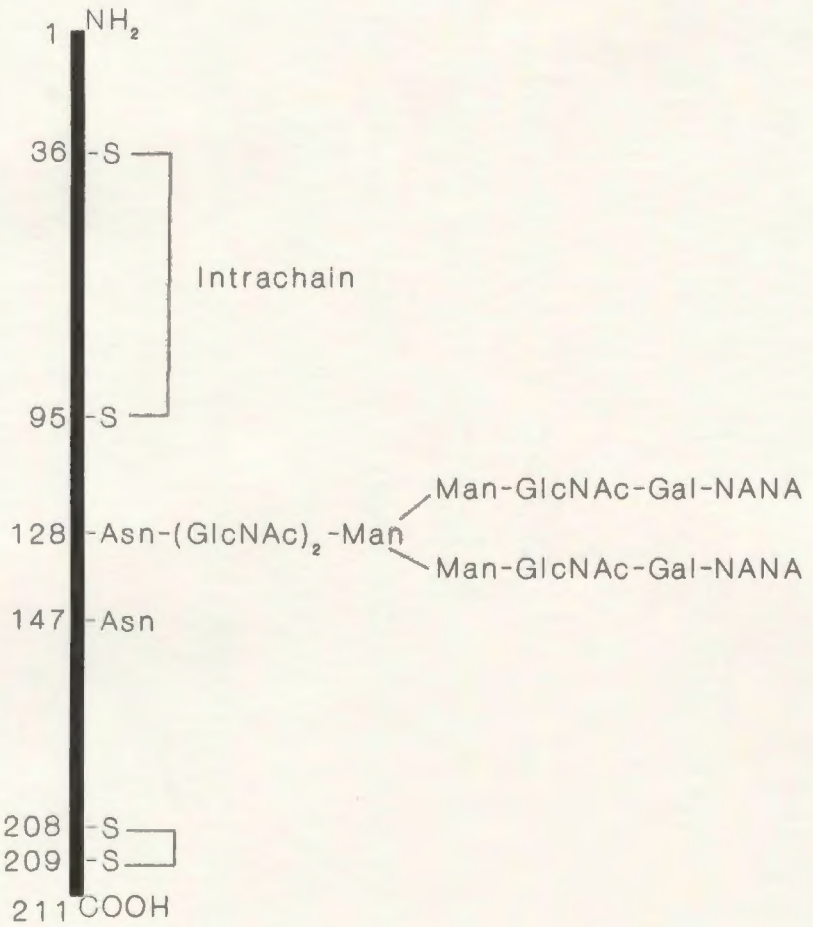
Rat CRP is a unique protein among CRPs of different species. Unlike CRP from human and rabbit, rat CRP is a normal component of rat serum (0.5-0.6 mg/ml) and a moderate acute phase protein that increases only 2 fold in response to injury

(Nagpurkar and Mookerjea, 1981; De Beer et al., 1982a). Although pentameric and composed of identical subunits, rat CRP contains an inter-chain disulphide bridge linking two of its five subunits. The remaining three subunits are non-covalently associated. This is in contrast to human and rabbit CRP in which all five subunits are held together non-covalently. Molecular cloning and amino acid analysis have revealed each of the subunits of rat CRP to be composed of 211 amino acids and each subunit having a molecular weight of approximately 26 kDa (Rassouli et al., 1992). However, a distinctive feature of rat CRP, not found in the human CRP amino acid sequence, is the presence of a heptapeptide sequence at the C-terminal of the molecule (Fig. 2). This sequence contains two cysteine residues that are believed to be responsible for inter-chain disulphide bonding between two of the rat CRP subunits. Nevertheless, the remaining amino acid sequence of rat CRP shares extensive identity (~60 %) with human CRP.

Unlike human and rabbit CRP, rat CRP is a glycoprotein. Rat CRP contains 18 % carbohydrate, composed mainly of a N-linked complex-type bi-antennary chains with some evidence of a small amount of tri-antennary chains. Glycosylation sites exist on each of the five subunits of rat CRP at position Asn-128 (Rassouli et al., 1992; Sambasivam et al., 1992). The presence of N-acetyl-neuraminic acid in the covalently-bound carbohydrate on rat CRP, together with the high content of Asp and Glu relative to the content of basic amino acids (Rassouli et al., 1992), both account for the acidic nature of this protein (pI 3.8).

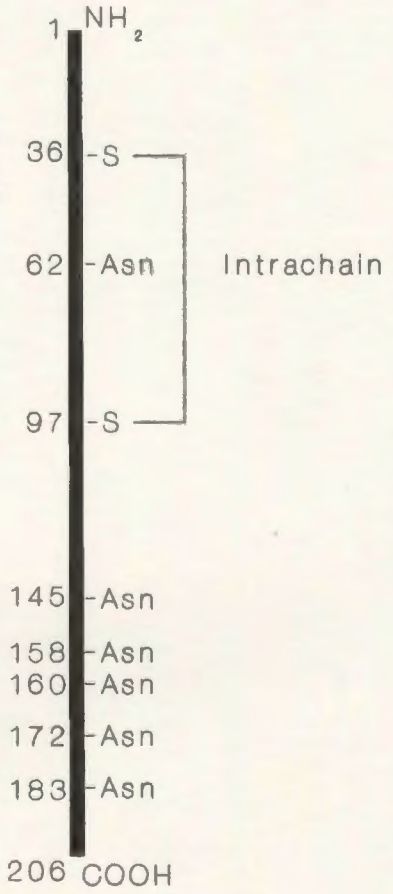
Fig. 2. **Comparison of the primary structure of rat CRP with human CRP.** This figure was constructed using the results of Rassouli et al. (1992) and Sambasivam et al. (1992) for rat CRP, and Lei et al. (1985) for human CRP.

Rat CRP
(MW 125K; pI 3.8); 5 subunits



Asn 128 & Asn 147:
potential N-glycosylation sites (Asn-X-Ser) unique to rat CRP. Asn 128 is glycosylated with a complex type bi-antennary structure

Human CRP
(MW 120K; pI 6.4); 5 subunits



No potential glycosylation sites.

C. Binding Properties

Unlike rabbit and human CRP which bind to five molecules of phosphorylcholine, rat CRP can only bind to three molecules of phosphorylcholine but with affinity similar to that of human CRP for phosphorylcholine (Rassouli et al., 1992; Anderson et al., 1978). It is possible that the three non-covalently held subunits of rat CRP are involved in the binding to phosphorylcholine.

Rat CRP also binds to multilamellar liposomes composed of PC. This property of rat CRP appears to be specific to phosphorylcholine-moieties on the surface of the liposomes and can be inhibited by phosphorylcholine (Nagpurkar et al., 1983). The binding of rat CRP to PC is much greater than that to PS or phosphatidylethanolamine (PE).

D. Biological Properties

One of the first recognised biological properties of rat CRP, was its ability to inhibit the calcium-dependent heparin-lipoprotein precipitation reaction, a property not shared by rabbit and human CRP (Nagpurkar and Mookerjea, 1981). Later studies revealed the ability of rat CRP to selectively bind to lipoproteins containing apoproteins B and E (Saxena et al., 1987) in a manner that was calcium-sensitive and this binding could be inhibited by phosphorylcholine. These findings compare with those showing human and rabbit CRP to bind specifically to lipoproteins containing apoprotein B (De Beer et al., 1982b; Rowe et al., 1984a).

The binding of rat CRP to LDL, which involves the phosphorylcholine-binding domain of rat CRP, results in inhibition of the binding of LDL to receptors on liver cell membranes from estradiol-treated rats (Saxena et al., 1986).

It has recently been shown that a galactose-specific receptor activity on the surface of rat liver macrophages is identical to membrane-bound rat CRP (Kempka et al., 1990; Kolb-Bachofen, 1991a). This receptor mediates effective uptake of particulate material expressing multiple galactosyl groups on their surfaces (Kolb-Bachofen, 1991b), thus providing direct proof that rat CRP triggers phagocytic events in macrophages. Furthermore, this receptor is recycled and can be substituted with heterologous human CRP while maintaining functional capacity (Kolb-Bachofen, 1991a).

The binding of rat CRP to macrophages is also believed to result in internalization and degradation of the bound CRP by lysosomes. This may indicate a role for rat CRP in the clearance of pathogens bearing the phosphorylcholine ligand (Nagpurkar et al. 1992). This reaction also compares well with the ability of human CRP to bind to phagocytic cells and undergo proteolytic degradation.

Rat CRP can bind to rat hepatocytes. *In vitro* studies have shown that relatively large amounts of asialo-rat CRP, rat CRP and rabbit CRP bind to isolated rat hepatocytes, predominantly via the phosphorylcholine-binding domain on these proteins (Yang et al., 1992). It was also suggested that this binding was the result of disruption of the hepatocyte membrane. However, the *in vivo* clearance of asialo-rat

CRP, but not native rat CRP or rabbit CRP, occurs via the asialoglycoprotein receptors on the liver.

Section IV. CRP-Like Proteins

A. Serum Amyloid P-Component (SAP)

CRP is structurally related to another group of proteins, called serum amyloid P-component (SAP), which bind to carbohydrate moieties (Osmand et al., 1977). SAP was first identified as a component of amyloid deposits in humans (Cathcart et al., 1965) and was later described at a pentraxin (Osmand et al., 1977). In humans, SAP consists of 10 subunits, is a glycoprotein and is not an acute phase protein. SAP shares about 50% amino acid sequence identity with human CRP (Oliveira et al., 1979; Anderson and Mole, 1982).

SAP exhibits a Ca^{2+}-dependent binding to the pyruvate moiety of agarose (Pepys and Baltz, 1983; Skinner and Cohen, 1988) but does not bind to phosphorylcholine.

SAP has been found in almost all vertebrate species examined, including rabbit, rat, mice and human (Pepys et al., 1978a). It is not uncommon for a given species to have both SAP- and CRP-like properties in one molecule (Kilpatrick and Volanakis, 1991).

B. Syrian Hamster Female Protein (FP)

In syrian hamsters, a serum protein analogous to CRP is expressed in high levels only in females (Coe, 1977, 1982). Due to its sex-related distribution, this protein is designated as hamster female protein (FP). Unlike human CRP, FP is glycosylated and is only expressed in males during inflammation or following castration. In the normal adult female syrian hamster, this protein is present at high serum levels in the range of 0.7-3.0 mg/ml, while in normal males the level is much less (0.01-0.02 mg/ml). FP has a molecular weight of about 150 kDa, and is assembled non-covalently into a pentagonal arrangement (Coe, 1982).

The hamster FP has a binding affinity for both phosphorylcholine and agarose-in the presence of calcium (Coe, 1983). It has also been shown to be a component of hamster amyloid deposits (Coe, 1983). The amino acid sequence of the FP has 69% identity to human SAP and 50% identity to human CRP (Dowton et al., 1980).

C. Other Phosphorylcholine-Binding Proteins

1. Anti-phosphorylcholine myeloma proteins

Like CRP, anti-phosphorylcholine myeloma proteins have a phosphorylcholine-binding property (Pollet and Edelhoch, 1973). The anti-phosphorylcholine myeloma proteins, however, show much less binding specificity for the phosphate-moiety of the ligand than CRP (Young and Williams, 1978). Certain anti-phosphorylcholine myeloma proteins also bind to CPS and ß-lipoproteins (Leon and Young, 1971) and

inhibit platelet aggregation (Fiedel et al., 1976).

2. Perforin

Perforin, a pore-forming protein in cytolytic T-lymphocytes, has been demonstrated to bind to phosphorylcholine in a calcium-dependent manner. Phosphorylcholine is believed to be a receptor molecule for perforin (Tschopp et al., 1989).

Section V. Purpose of Present Research

One of the least understood effects of CRP is the inhibition of platelet aggregation. Although, several studies in the past have described the inhibitory effect of rabbit and human CRP on platelet aggregation, very little has been reported on the mechanism by which CRP inhibits platelet aggregation. It is, therefore, important to study these mechanisms in platelets. No previous study has examined the effect of rat CRP on platelet aggregation. Rat CRP merits special consideration since it differs from rabbit and human CRP with respect to its a) glycosylation status and b) concentration in the blood. Hence, the purpose of this investigation was to examine the effect of rat CRP on various aspects of platelet function such as aggregation and secretion of serotonin. This thesis also attempts to elucidate the mechanisms involved in the regulation of platelet function by rat CRP.

In order to elucidate the mechanism of action of CRP on platelets, it was

important to demonstrate a) the effect of rat CRP on platelet aggregation and b) the binding of rat CRP to platelets. It has been assumed that the binding of rat CRP to platelets may in turn affect the complex biological pathways involved in the regulation of platelet function. Because of the presence of PC-PLC in platelets and its possible role in the regulation of platelet function, the effect of rat CRP on PC-PLC and other platelet phospholipases were investigated.

CHAPTER 4

METHODS AND MATERIALS

Section I. Materials

A. Animals

1. Rats

Male Sprague-Dawley rats (body weight 250-300g) were obtained from Canadian Hybrid Farms, Centerville, Nova Scotia and were fed Purina rat chow *ad libitum* (Ralston-Purina of Canada Ltd., Don Mills, Ontario).

2. Rabbits

Male New Zealand white rabbits (body weight 1.5-3.5 kg) were purchased from Memorial University's Animal Vivarium, St. John's, Newfoundland and were fed rabbit chow *ad libitum* (Robinhood Multifoods Inc., St. John's, Newfoundland).

B. Chemicals and Reagents

Unless otherwise specified, chemicals and reagents were of commercial origin and were of the highest grade available.

Choline chloride was purchased from J.T. Baker Chemical Co., New Jersey, USA. Phospholipase C (from *Cholistritium welchii*; 10.6 U/mg protein),

phospholipase A_2 (from *Naja naja* venom; 2000 U/mg protein), creatine phosphokinase (30 U/mg protein), thrombin (bovine; 610 NIH units/mg), prostaglandin I_2, prostaglandin E_1, imipramine, creatine phosphate, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), glyceryl-3-phosphocholine, palmitic acid, CDP-choline, PA, DAG, myristic acid, thromboxane B_2, HETE, and HHT, Triton X-100, oleic acid, LPC, synthetic PAF (1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) (2 mg/ml in chloroform), bovine serum albumin (BSA), rat fibrinogen, α_1-acid glycoprotein (bovine), human CRP, ADP, arachidonic acid, PC, PS, PE, PI, and phosphorylcholine chloride (calcium salt) were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Sodium deoxycholate was obtained from Fisher Scientific Co., New Jersey, USA. Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenyl-glycouril) was from Pierce, Rockford, Il., USA. Cyanogen bromide (CNBr)-activated sepharose 4B was purchased from Pharmacia, Baie D'Urte, Quebec.

C. Radioisotopes

1-Palmitoyl-2-[9,10-^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine ([2-^{3}H palmitoyl]DPPC; 32.9 Ci/mmol), 1,2-dipalmitoyl-*sn*-glycerol-3-[^{3}H-*methyl*]phosphocholine ([^{3}H-choline]DPPC; 37 Ci/mmol), (myo-inositol-2-^{3}H(N))-phosphatidylinositol (phosphatidyl[^{3}H]inositol; 5.2 Ci/mmol), myo-[2-^{3}H(N)]-inositol ([^{3}H]inositol; 12.3 Ci/mmol), Na^{125}I (reductant free, 2 mCi in 4-5 μl 0.1 M NaOH), and [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid ([^{3}H]arachidonic acid; 76.0

Ci/mmol) were products of Dupont, Canada. 5-[^{14}C]Hydroxytryptamine ([^{14}C]serotonin; 57 mCi/mmol), (1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl)-phosphatidylcholine ([2-^{3}H arachidonyl]PC; 120 Ci/mmol), 1-O-[^{3}H]alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (mixture of C-16 and C-18 alkyl ethers) ([^{3}H-alkyl]PAF; 81 Ci/mmol), [^{14}C *methyl*]choline (55 mCi/mmol), and [9,10(N)-^{3}H]myristic acid ([^{3}H]myristic acid; 54 Ci/mmol) were purchased from Amersham. [N-*methyl*-^{14}C]Lyso-PAF (55 mCi/mmol) was from American Radiochemical Corporation, St. Louis, Mo., USA.

D. Buffers and Solutions

Tyrode Solution: 137 mM NaCl, 2.6 mM KCl, 0.9 mM $MgCl_2$, 5.5 mM D-glucose, 0.25% (w/v) BSA, 12 mM $NaHCO_3$ and 0.4 mM NaH_2PO_4 (pH 7.4).

Calcium-Free Tyrode Solution: 137 mM NaCl, 2.6 mM KCl, 0.9 mM $MgCl_2$, 5.5 mM D-glucose, 1 mM EGTA, 0.25% (w/v) BSA, and 12 mM $NaHCO_3$ (pH 6.5).

Tyrode-HEPES Solution: 137 mM NaCl, 2.6 mM KCl, 0.9 mM $MgCl_2$, 5.5 mM D-glucose, 0.25% (w/v) BSA, and 5 mM HEPES (pH 7.4).

3.6% Citrate Anticoagulant: 85 mM Trisodium citrate, 70 mM citric acid, and 110 mM D-glucose.

Citrate-Buffered Platelet Washing Solution: 137 mM NaCl, 11 mM D-glucose, 0.25% (w/v) BSA, 6 mM sodium citrate, and 4.8 mM citric acid (pH 6.5).

Tris-HCl Buffered Platelet Washing Solution: 1.5 mM EDTA, 125 mM NaCl, 4 mM D-glucose, and 12 mM Tris-HCl (pH 7.4).

E. Antiserum

Rabbit-raised antiserum to rat CRP was prepared as described by Nagpurkar and Mookerjea (1981). Goat-raised antiserum to rabbit CRP was a gift from Dr. H. Gewurz, Department of Immunology, Rush Medical College, Chicago.

Section II. Induction of Inflammation in Rabbits

Acute inflammation was induced in rabbits by subcutaneous injection of commercial grade turpentine (0.5 ml/kg body weight) into the dorsolumbar region. Blood samples was taken at 48 h after injection from the marginal ear artery. Rabbits were also bled by cardiac puncture under anaesthesia induced by sodium pentabarbital (1 ml/kg body weight). The serum from inflamed rabbits was checked for the presence of rabbit CRP by immunodiffusion analysis using antiserum to rabbit CRP.

Section III. Preparative Procedures

A. Preparation of Sepharose-Phenylphosphorylcholine Affinity Adsorbent

Sepharose-phenylphosphorylcholine affinity adsorbent was prepared as described by Nagpurkar and Mookerjea (1981) which involved reduction of *p*-nitrophenylphosphorylcholine with H_2 and coupling the reduced product to CNBr-activated sepharose 4B. The resulting affinity adsorbent was stored in 5 mM Tris-HCl buffer (pH 7.4) containing 0.01% sodium azide, at 4 °C.

B. Isolation and Purification of Rat and Rabbit CRP

1. Rat CRP

Rat CRP was isolated from the serum of male Sprague-Dawley rats (300-450g) using sepharose-phenylphosphorylcholine affinity adsorbent equilibrated with 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl as described by Nagpurkar and Mookerjea (1981). The protein concentration was determined by the method of Lowry et al. (1951). The purity of the protein was determined by SDS-PAGE using the Phastsystem (Pharmacia), and by high performance liquid chromatography (HPLC) gel filtration column (TSK-250 gel filtration column, 7.5 x 300 mm; Bio-Rad). Preparations with purity greater or equal to 97%, according to HPLC profile, were stored at -20 °C for use in experiments.

2. Rabbit CRP

Rabbit CRP was isolated from serum of inflamed rabbits following a procedure similar to that used in the isolation of rat CRP, except that 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, and 2.5 mM $CaCl_2$ was used in purification of this protein. The isolated rabbit CRP was determined to be free of other proteins by analysis on SDS-PAGE and HPLC. The identity of the purified rabbit CRP was confirmed by immunodiffusion analysis using antiserum to rabbit CRP and was stored at -20 °C for use in experiments.

C. Preparation of Platelet Sonicates

1. Preparation of Washed Platelets

Rat blood was collected via the abdominal aorta of ether-anaesthetized rats into plastic syringes containing 0.1 vol of 3.6 % citrate anticoagulant. Rabbit blood was collected from the central ear artery into plastic syringes containing 0.1 vol of 3.6 % citrate anticoagulant. Human blood (4.5 mL) was collected in silicone coated tubes containing 0.5 ml of 0.129 M buffered citrate solution (16.0 mg sodium citrate dihydrate, 2.1 mg citric acid monohydrate) from healthy volunteers who had denied taking any drugs for at least 1 week prior to donation.

Platelet rich plasma (PRP) was isolated as the supernatant resulting after centrifuging (220 x g) the blood for 8-10 min at 22 °C.

PRP was diluted 1:1 with Tris-HCl buffered platelet washing solution (pH 7.4)

containing 2 mM phosphorylcholine and centrifuged (900 x g) for 10 min at 22 °C to sediment platelets (modified from Choudhury et al. 1987). The platelet pellet was re-suspended, by gentle agitation, into fresh washing solution without 2 mM phosphorylcholine and re-centrifuged (900 x g) for 10 min. This step was repeated 2 times and the platelet pellet was finally re-suspended in a solution of 25 mM Tris-HCl buffer (pH 7.4) containing 125 mM NaCl, and 2.5 mM $CaCl_2$.

The platelet concentration was determined by microscopy using a counting chamber (1/400 mm^2 x 1/10 mm deep) from Hausser Scientific Pa. and the platelets were diluted to a concentration of 5 x 10^8/ml. Platelets were then sonicated on ice, 3x for 30 sec, using a Branson Sonifier fitted with a microtip at 60 Watts (Hayakawa et al., 1988). In some cases the concentration of protein in the platelet sonicates was determined by the method of Lowry et al. (1951).

2. Fractionation of Platelet Sonicates

Rat platelet sonicates were fractionated by centrifuging (105,000 x g) in a Beckman Type 40 rotor for 60 min at 4 °C using a Beckman Model L3-50 Ultracentrifuge (Hayakawa et al., 1988). The supernatant of the centrifuged sonicates was removed and was representative of a soluble fraction. The pellet was re-suspended in a volume equal to the volume of the supernatant, by agitation and sonication for three 5 sec intervals to give a re-suspended particulate fraction.

D. Radioiodination Procedures

Rat CRP was radioiodinated by the iodogen method (Pierce) utilizing carrier-free $Na^{125}I$. Rat [^{125}I]CRP was purified by chromatography on a Sephadex G-25 Medium column (20 ml bed volume; Pharmacia) and a Sepharose-phenylphosphorylcholine affinity column, and typically had a specific activity of 6.1 x 10^5 cpm/μg (2.8 μCi/μg) or greater. Rat [^{125}I]CRP was diluted with unlabelled CRP to give the working concentrations.

Section IV. Analytical Procedures

A. Electrophoresis

The Phastsystem electrophoresis unit (Pharmacia) was used to analyze CRP samples by SDS-PAGE. Typically rat or rabbit CRP at concentrations of 1-2 mg/ml were analyzed. Electrophoresed samples were stained with Coomassie Brilliant Blue R 250 dye (Pharmacia Blue R) by the development unit of the Phastsystem. A typical SDS-PAGE of purified rat and rabbit CRP is shown in Fig. 1.

B. High Performance Liquid Chromatographic (HPLC) Analysis

The purity of rat and rabbit CRP, isolated from sepharose-phenylphosphorylcholine affinity columns, was determined on a Perkin-Elmer series 4 HPLC system using a TSK-250 gel filtration column (7.5 x 300 mm; Bio-Rad).

Fig. 1. **SDS-PAGE of purified rat and rabbit CRP.** SDS-PAGE was performed on 7.5% polyacrylamide gels (Pharmacia) using 2.5% SDS buffer strips (Pharmacia) by Phastsystem (Pharmacia). Lane #1 and #8 are low molecular weight standards (Pharmacia), lanes #2 and #7 do not contain any sample, lane #3 is rabbit CRP treated with ß-mercaptoethanol, lane #4 is rabbit CRP (untreated), lane #5 is rat CRP treated with ß-mercaptoethanol, and lane #6 is rat CRP (untreated). The low molecular weight standards are phosphorylase b (94 kDa; A), BSA (67 kDa; B), ovalbumin (43 kDa; C), carbonic anhydrase (30 kDa; D), trypsin inhibitor (20.1 kDa; E), and α-lactalbumin (14.4 kDa; F).

Protein samples were filtered through 0.45 μm filters to remove particulate matter. Typically 10-50 μg of protein were injected to the column, which had been equilibrated with 10 bed volumes of 0.05 M Na_2SO_4, 0.02 M NaH_2PO_4 buffer (pH 6.8) at a flow rate of 1.0 ml/min. The eluent from the column was continuously monitored at 280 nm using a LC-95 UV/visible spectrophotometer detector (Perkin-Elmer). The area under the absorbance peak from eluted protein was determined using a LCI-100 Laboratory Computing Integrator (Perkin-Elmer) and area % was reported as % purity of protein. Typical HPLC profiles for rat and rabbit CRP are shown in Fig. 2.

C. Thin Layer Chromatography (TLC)

TLC was used to separate DAG, choline metabolites, PA, or various phospholipids from other lipids. This involved the application of aliquots of lipid dissolved in chloroform/methanol solvent or aliquots of the aqueous layers of extracted samples to Whatman K5 silica gel 150A TLC plates (layer thickness of 250 μm) for chromatography. In each case, except when choline metabolites were measured, a large strip of filter paper was placed in each TLC development chamber to aid in saturation of the chamber atmosphere with the mobile phase.

1. Separation of Choline Metabolites

Identification of the aqueous soluble products of PC metabolism was made by

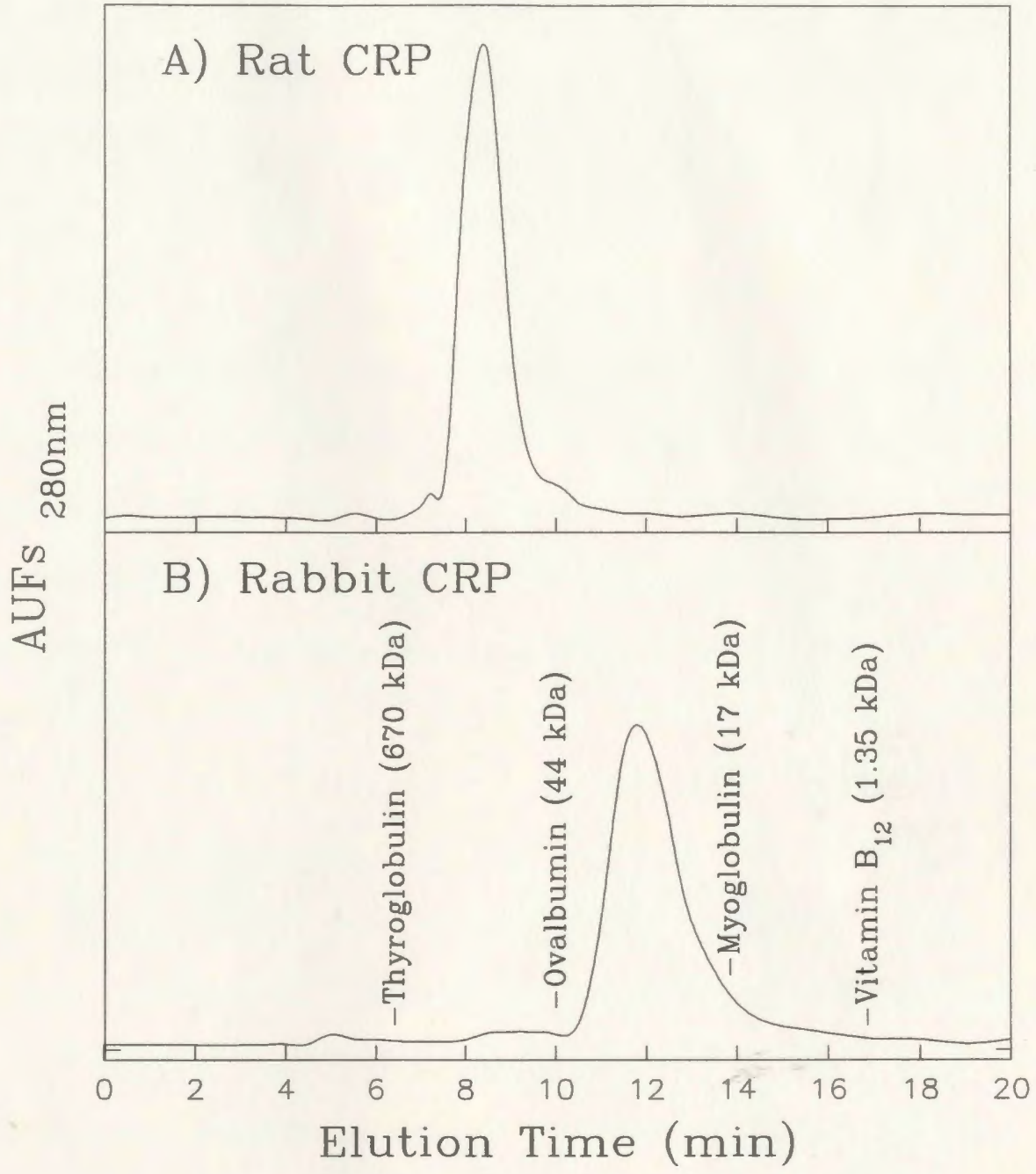


Fig. 2. **HPLC profile of purified rat and rabbit CRP.** A) rat (20 μg) or B) rabbit CRP (20 μg) in a final volume of 20 μl was applied to a HPLC gel filtration column. Trace represents the absorbance detected at 280 nm by LC-95 UV/Visible Spectrophotometer Detector as CRP was eluted from the column.

applying (50-400 μl) aliquots of the aqueous layers of extracted samples to Whatman K5 silica gel 150A TLC plates and developing them in methanol/0.9% NaCl/ammonium hydroxide (10/10/1, v/v/v) to separate water soluble choline metabolites (Vance et al., 1980). Authentic standards consisting of DPPC (5 μg), LPC (5 μg), glyceryl-3-phosphocholine (20 μg), CDP-choline (20 μg), phosphorylcholine (20 μg) and choline (20 μg) were chromatographed with the aqueous extract. After development, the plates were air dried and were stained with iodine vapour to aid in localization of the products. Radioactive products were scraped from the plates into vials containing 10 ml of Ready Safe liquid scintillation cocktail (Beckman) and counted by Wallac 1209 Rackbeta Liquid Scintillation Counter.

2. Separation of Phospholipids

To measure incorporation of [^{3}H]myristic acid or [^{3}H]arachidonic acid into platelet lipids, re-suspended lipid was applied to Whatman K5 silica gel 150A TLC plates and developed in chloroform/methanol/H_2O (65/25/4; v/v/v) (Rouser et al., 1976). This solvent system allows separation of neutral lipids from phospholipids. PC and PE are well separated from other lipids. PS and PI, however, have similar mobilities in this system and were, therefore, quantified as one unit. Authentic standards consisting of PC (5 μg), PE (5 μg), PI (5 μg), LPC (5 μg), PS (5 μg), PA (10 μg), DAG (5 μg) and fatty acids (10 μg) were chromatographed with each sample

to confirm adequate separation of metabolites. After air drying the plates, the products were visualized by staining with iodine vapour. Radioactive lipids were scraped from the plate and were counted for radioactivity.

3. Separation of Arachidonic Acid Metabolites

The solvent used for separation of arachidonic acid and its lipoxygenase and cycloxygenase products was the upper phase of ethylacetate/2,2,4-trimethylpentane/acetic acid/H_2O (9/5/2/10, v/v/v/v) which gave good separation of phospholipids, PA, arachidonic acid and its metabolites (Billah et al., 1980). Authentic arachidonic acid (10 μg), thromboxane B_2 (1 μg), HHT (0.5 μg), HETE (0.5 μg), PA (10 μg) and PC (5 μg) standards were chromatographed with each sample to aid in identification of the labelled metabolites. After developing the plate the various lipid standards were visualized by iodine vapour. Radioactive products were scraped from the plate and counted for radioactivity.

4. Separation of DAG

In PC-PLC assays using [2-^{3}H palmitoyl]DPPC as substrate, the identification and separation of DAG was made from aliquots of the non-aqueous layers of extracted samples that were removed and applied to silica gel 60 TLC plates (layer thickness of 250 μm; Merck). The plates were developed in hexane/diethylether/90% formic acid (60/40/1, v/v/v) to isolate [^{3}H]DAG (Huang and Cabot, 1990). Authentic

standards consisting of DPPC (5 μg), LPC (5 μg), PA (10 μg), palmitic acid (10 μg) and DAG (5 μg) were chromatographed with each sample to aid in identification of the labelled metabolites. The lipids on the developed plates were stained with iodine vapour. The stained lipids were scraped from the plate and counted for radioactivity.

To measure formation of DAG from platelets labelled by [^{3}H]myristic acid, re-suspended lipid was applied to Whatman K5 silica gel 150A TLC plates and developed in hexane/diethylether/90% formic acid (60/40/1, v/v/v). In this case authentic standards consisting of PC (5 μg), LPC (5 μg), PA (10 μg), DAG (5 μg), and myristic acid (10 μg) or arachidonic acid (10 μg) were also chromatographed on each plate as standards, and stained by iodine vapour. The stained lipids were scraped from the plate and counted for radioactivity.

5. Separation of PA

In PC-PLC assays using [2-^{3}H palmitoyl]DPPC as substrate, the extracted lipids suspended in chloroform/methanol (1/1, v/v) was applied to silica gel 60 TLC plates (layer thickness of 250 μm; Merck) and developed in chloroform/pyridine/70% formic acid (50/25/7, v/v/v) to separate PA from other lipid (Huang and Cabot, 1990). Plates were allowed to air dry for at least 24 hours to evaporate the solvent, before staining with iodine vapour. In this case authentic standards consisting of PC (5 μg), LPC (5 μg), palmitic acid (10 μg), PA (10 μg) and DAG (5 μg) were also chromatographed with each sample, and stained with iodine vapour. The stained

lipids were scraped from the plate and counted for radioactivity.

Section V. Platelet Function Studies

A. Platelet Aggregation Studies using PAF or ADP

1. Preparation of Platelet Rich Plasma

Rat PRP was prepared from blood using 0.1 vol of 90 mM EGTA in 0.35 % (w/v) NaCl as anticoagulant. PRP was prepared by centrifuging (220 x g) blood for 8-10 min at 22 °C. Human PRP was isolated from 4.5 ml portions of blood collected in silicone coated tubes containing 0.5 ml of 0.129 M buffered citrate solution (16.0 mg sodium citrate dihydrate, 2.1 mg citric acid monohydrate) from healthy volunteers who had denied taking any drugs for at least 1 week prior to donation. PRP was isolated from New Zealand white rabbit blood using 0.1 vol of 3.6 % citrate anticoagulant.

Platelet poor plasma (PPP) was prepared by centrifuging (900 x g) blood minus PRP for 10 min at 22 °C. In cases where platelet aggregation was to be measured in PRP, the platelet concentration was diluted to 4 x 10^8/ml using PPP.

2. Preparation of Washed Platelets

Washed rat platelets were prepared from PRP by washing 2x in a solution containing 2 mM phosphorylcholine and 2x in a solution without phosphorylcholine by a method similar to that described in section III.C.1. The washed platelets were

re-suspended, by gentle agitation, in 16 mM NaH_2PO_4 buffer (pH 7.4) containing 125 mM NaCl, 5 mM KCl, and 4 mM D-glucose and diluted to a concentration of 4 x 10^8/ml. No rat CRP was detected on the platelets by immunodiffusion analysis against antiserum to rat CRP.

Rabbit platelets were isolated from PRP by centrifugation (900 x g) for 10 min at 22 °C. The platelet pellet was then washed twice in citrate-buffered platelet washing solution (pH 6.5) as described by Vigo (1985). The washed platelets were finally re-suspended in Tyrode solution (pH 7.4), counted, and diluted to 4 x 10^8/ml to be used in platelet aggregation studies.

3. Platelet Aggregation Assays

Platelet aggregation was monitored in a dual channel Chrono-log aggregometer (Model 440) in siliconized cuvettes containing PRP, or suspension of washed platelets taken from rat, rabbit or human using a previously published procedure (Choudhury et al. 1987). To measure PAF- or ADP-induced platelet aggregation, aliquots of the appropriate platelet suspension (concentration of 4 x 10^8/ml), were added to siliconized cuvettes and continuously stirred. Varying amounts of either rat CRP, other proteins or the antiserum to rat CRP were added to the cuvettes and equilibrated at 37 °C in presence of 1.5 mM $CaCl_2$. Finally platelet agonist, ADP (44 μM) or PAF (0.5 μM), was added to the platelets and the amount of light transmitted through the stirred platelet suspension was recorded. PPP or the appropriate platelet

suspension buffer was used as a blank to represent 100 % light transmission.

B. Platelet Aggregation and [^{14}C]Serotonin Secretion Studies using Thrombin as Agonist

1. Preparation of [^{14}C]Serotonin-Labelled Platelets

Blood was collected from the abdominal aorta of ether-anaesthetized male Sprague-Dawley rats, into plastic syringes containing 0.1 vol of 3.6 % citrate anticoagulant. Platelet rich plasma (PRP) was obtained by centrifuging (220 x g) blood for 8-10 min at 22 °C. The PRP was diluted 1:1 with calcium-free Tyrode solution (pH 6.5). Diluted PRP was centrifuged (900 x g) for 10 min at 22 °C to sediment the platelets. The platelet pellet was then re-suspended in calcium-free Tyrode solution (pH 6.5) and loaded with [^{14}C]serotonin following the method of Holmsen and Dangelmaier (1989), but with certain modifications. Briefly, the re-suspended platelets were incubated with [^{14}C]serotonin (0.1 μCi/ml) for 30 min at 22 °C to allow incorporation of the [^{14}C]serotonin into dense granules. Platelets were then washed (x2) by centrifugation (900 x g) for 10 min to remove un-incorporated label. The washed platelets were finally re-suspended into Tyrode solution (pH 7.4) and the platelet concentration was adjusted to 4 x 10^8/ml.

To determine incorporation of [^{14}C]serotonin into platelets an aliquot (100 μl) of the platelet suspension was transferred to a scintillation vial containing 10 ml of scintillation cocktail and counted for radioactivity. This labelling protocol usually

resulted in incorporation of approximately 50,000 DPM/4 x 10^8 platelets.

2. Platelet Aggregation and [^{14}C]Serotonin Secretion Assays

Platelets loaded with [^{14}C]serotonin were used to study thrombin-induced platelet aggregation and [^{14}C]serotonin secretion. The protocol for aggregation was similar to that described in section V.A.3. [^{14}C]Serotonin secretion was measured as described by Holmsen and Dangelmaier (1989) with certain modifications. Briefly, [^{14}C]serotonin-loaded platelets (4 x 10^8/ml; 50,000 DPM/ml) in Tyrode solution (pH 7.4) were incubated with 1.5 mM $CaCl_2$ for 15 min either in the absence or presence of rat CRP. Imipramine (2 μM) was added to platelets prior to stimulation to inhibit re-uptake of released serotonin. Thrombin (0.05-2.0 U/ml) was finally added to the stirred platelets and the aggregation was monitored for 3 min in a dual-channel Chrono-log aggregometer (model 440). The final volume of assays after adding thrombin were either 500 μl or 1 ml. The extent of aggregation was expressed as the percent increase in light transmission after the addition of thrombin. To determine the secretion of [^{14}C]serotonin from the platelets, a 100 μl aliquot of the platelet suspension was transferred from the aggregometer to an eppendorf microfuge tube containing 25 μl of ice-cold formaldehyde (0.633 M) and EDTA (50 mM). The mixture was centrifuged at 12,800 x g for 1 min and an aliquot of the supernatant containing [^{14}C]serotonin was counted for radioactivity. The [^{14}C]serotonin secreted from activated platelets was calculated as a percentage of the total radioactivity in 4 x

10^8 platelets/ml.

Section VI. Platelet Binding Studies

A. Preparation of Washed Platelets

1. Rat Platelets

Washed platelets were prepared from rat PRP containing 3.6% citrate anticoagulant following the protocol described in section V.A.2. and finally re-suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 % (w/v) BSA, 0.15 M NaCl, and 5 mM $CaCl_2$ to be used for CRP-platelet binding studies. No CRP was detected on the platelets by immunodiffusion analysis against antiserum to rat CRP.

2. Rabbit Platelets

Washed rabbit platelets were prepared as described in section V.A. for rabbit platelets and finally re-suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 % (w/v) BSA, 0.15 M NaCl, and 5 mM $CaCl_2$ to be used for CRP-platelet binding studies.

3. Human Platelets

Washed human platelets were prepared from PRP after diluting 1:1 with citrate-buffered platelet washing solution (pH 6.5) and centrifuging (900 x g) for 10 min at 22 °C. The sedimented platelets were then re-suspended in the same buffer

and washed as described for rabbit platelets in section V.A. The washed platelets were finally suspended in 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 % (w/v) BSA, 0.15 M NaCl, and 5 mM $CaCl_2$ to be used in CRP-platelet binding studies.

B. Binding Assays

Platelet binding studies were performed by incubating different amounts of rat [^{125}I]CRP (0.5-16.0 mg/ml) with washed platelets from rat, rabbit, or human. Binding studies were performed in 1.5 ml Eppendorf microfuge tubes which had been pre-soaked in a solution of 0.25 % (w/v) BSA. Rat [^{125}I]CRP was added to suspensions of freshly prepared washed platelets in 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 % (w/v) BSA, 0.15 M NaCl and 5 mM $CaCl_2$ in a final volume of 0.5 ml and incubated at 37 °C for 30 min in a shaking water bath. Immediately following incubation the reaction mixture was centrifuged (12,800 x g) for 2 min at 4 °C in a Beckman Microfuge which resulted in complete sedimentation of the platelets. The supernatant was carefully aspirated and the platelet pellet was washed (1x) in ice cold buffer. The tips of the tubes were cut off just above the pellet and the radioactivity was counted by a γ-counter (Beckman). Non-specific binding was measured in parallel incubations containing a 25-400 fold molar excess of unlabelled rat CRP. Specific binding of rat CRP to platelets was calculated by subtracting non-specifically bound CPM from the total CPM bound and was expressed as μg of rat CRP bound per 2 x 10^7 platelets.

Binding data was analyzed by means of the computer software program Enzfitter (Elsevier-Biosoft) using non-linear regression analysis. The binding data was used for Scatchard plots using linear regression analysis, and the binding parameters K_d and the number of binding sites were estimated using the equation:

$$\frac{\nu}{[CRP]} = \frac{N - \nu}{K_d}$$

Where the value ν is the average number of rat CRP molecules bound to each platelet. N represents the number of binding sites on each platelet for CRP and K_d is the dissociation constant.

Section VII. Binding of [^{3}H-alkyl]PAF to Rat CRP

To study the binding of [^{3}H-alkyl]PAF to rat CRP, a HPLC gel filtration method was used. Purified rat CRP (0.5 nmol) was pre-incubated with [^{3}H-alkyl]PAF (0.05 nmol; 81 Ci/mmol) in a final volume of 50 μl. The mixture was then applied to an equilibrated HPLC gel filtration column (TSK-Sperogel gel filtration column, 7.5mm x 300mm; Beckman) maintained at a flow rate of 0.8 ml/min. The equilibration buffer contained 50 mM Na_2SO_4, 20 mM NaH_2PO_4 (pH 6.8) in the presence or absence of 2.5 mM $CaCl_2$. Elution of the protein from the column was monitored at 280 nm by Perkin-Elmer LC-95 UV/Visible Spectrophotometer Detector. Elution of the [^{3}H-alkyl]PAF was synchronously monitored by a Beckman 171 Radioisotope Detector utilizing Ready Flow III liquid scintillation cocktail. The

column was subsequently washed between runs with 0.1% SDS in equilibration buffer without calcium to remove radioactivity remaining on the column.

Section VIII. Assays for Purified Phospholipases

A. Assay for PC-PLC from *C. welchii*

To measure PC-PLC activity in enzyme isolated from *C. welchii*, assays were carried out in incubation mediums containing 25 mM Tris-HCl buffer (pH 7.4), 125 mM NaCl, 2.5 mM $CaCl_2$ and 150 μM [^{3}H-choline]DPPC (1000-1500 DPM/μM) as substrate and maintained at 37 °C in a shaking water bath. In some experiments rat CRP was also incubated with the substrate before adding enzyme. Reactions were started by adding 0.02 U *C. welchii* PC-PLC/ml and typical reactions (0.5 ml final volume) were allowed to proceed for 2 min. Reactions were quenched by adding 3.75 vol of chloroform/methanol containing 2% acetic acid (1/2, v/v) and was extracted (Bligh and Dyer, 1959). The aqueous layer containing water soluble [^{3}H]phosphorylcholine, was counted for radioactivity. The identity of [^{3}H]phosphorylcholine as the released product was confirmed by TLC.

B. Assay for PLA_2 from *Naja naja* venom

PLA_2 assays, using enzyme isolated from *Naja naja* venom were carried out in standard incubation mediums containing 100 mM Tris-HCl buffer (pH 7.4), 5 mM

$CaCl_2$ and 160 μM [2-^{3}H palmitoyl]DPPC (1000 DPM/μM) as substrate. The final assay volume was 100 μl which included the enzyme and in some cases rat CRP. Reactions were started by adding 0.02 U *Naja naja* venom PLA_2/ml to the incubation medium, maintained at 37 °C in a shaking water bath, and allowed to proceed for 5 min. Reactions were terminated by adding 32.5 vol of chloroform/methanol/heptane (1.41/1.25/1.0, v/v/v) followed by 7.5 vol of 0.14 M borate buffer (pH 10.5) and 4 vol H_2O (Puolakkainen et al., 1987), and mixed by vortexing. The 2 layers were separated by centrifugation (2600 x g) for 20 min at 4 °C. The upper phase, containing the free [^{3}H]palmitic acid was counted for radioactivity.

Section IX. Assays for Phospholipases from Platelet Sonicates

A. Assay for PLA_2

The activity of PLA_2 in rat platelet sonicates was measured in a standard incubation medium containing 25 mM Tris-HCl buffer (pH 7.4), 125 mM NaCl, 2.5 mM $CaCl_2$, using 50 μM [2-^{3}H arachidonyl]PC (900 DPM/μM) as substrate. In typical assays the reaction was begun by adding platelet sonicates (8 x 10^7/ml; 50 μg protein/ml) to the incubation medium equilibrated at 37 °C in the absence or presence of rat CRP, in a final volume of 120 μl. The reaction was stopped after 20 min and lipids were extracted as described for snake venom PLA_2 assay. The upper phase, containing the [^{3}H]arachidonic acid was counted for radioactivity.

B. Assay for PI-PLC

The PI-PLC activity in rat platelet sonicates (2.5 x 10^7/ml; 15 μg protein/ml) was measured in a standard incubation medium containing 25 mM Tris-HCl buffer (pH 7.4), 125 mM NaCl, 2.5 mM $CaCl_2$, and 16 μM phosphatidyl[^{3}H]inositol (3050 DPM/μM) and 6.4 μM PE as substrate (final volume of 100 μl) in the presence or absence of rat CRP. This procedure was modified from a previously described assay for PI-PLC using sonicated human platelets (Bleasdale et al., 1990). Reactions were allowed to proceed for 3 min at 37 °C after adding the platelet sonicates. Reactions were quenched by adding 3.75 vol of chloroform/methanol containing 2% acetic acid (1/2, v/v) and extracted (Bligh and Dyer, 1959). A portion of the aqueous layer which contained water soluble [^{3}H]inositol phosphates were added to 10 ml of Ready Safe liquid scintillation cocktail and counted for radioactivity.

C. Assay for PC-PLC

The PC-PLC activity in rat, rabbit or human platelet sonicates (5 x 10^7 cells/ml; 30 μg/ml) were measured in standard incubation mixtures containing 25 mM Tris-HCl buffer (pH 7.4), 125 mM NaCl, 2.5 mM $CaCl_2$, tritium-labelled substrate, (18 μM; 10,000-30,000 dpm/μM) [^{3}H-choline]DPPC or [2-^{3}H palmitoyl]DPPC, in the presence or absence of rat CRP in a final volume of 400 μl. Incubations were carried out for 20 or 40 min at 37 °C in a shaking water bath. Reactions were stopped by adding 1.875 ml of a mixture of chloroform/methanol (1/2, v/v) containing 2% acetic

acid. This was immediately followed by the addition of 100 μl of phosphorylcholine/choline (20 mg/ml each) as internal standards. The assays were extracted (Bligh and Dyer, 1959) and centrifuged (1000 x g) for 15 min to separate aqueous and non-aqueous layers. Aliquots of aqueous layers containing [^{3}H]phosphorylcholine were added to 10 ml of Ready Safe scintillation cocktail and counted for radioactivity.

In certain cases, aqueous layers or organic layers were analyzed by TLC for formation of [^{3}H]DAG or for formation of [^{3}H]phosphorylcholine.

D. Choline Kinase Activity

Choline kinase assays were carried out using rat platelet sonicates in order to determine if choline kinase was responsible for the [^{3}H]phosphorylcholine that was formed by platelet sonicates in PC-PLC incubation medium, containing [^{3}H-choline]DPPC. The standard incubation mixture contained 25 mM Tris-HCl buffer (pH 7.4), 125 mM NaCl, 2.5 mM $CaCl_2$, 0.6 μM [^{14}C-*methyl*]choline as substrate and was equilibrated at 37 °C. Platelet sonicates (5 x 10^7 cells/ml; 30 μg protein/ml) were added to the incubation mixture to give a final volume of 400 μl. Reaction was stopped after 20 min by adding 1.875 ml of chloroform/methanol (1/2, v/v) containing 2% acetic acid and 100 μl of a mixture of phosphorylcholine/choline (20 mg/ml each). The assays were extracted (Bligh and Dyer, 1959) and the aqueous layer was chromatographed by TLC. Bands corresponding to phosphorylcholine and

choline were scraped and counted as described for the PC-PLC assay using platelet sonicates.

Section X. Assays for Phospholipases in Intact Platelets

A. Isolation of Platelets from PRP

Blood was collected from the abdominal aorta of ether-anaesthetized male Sprague-Dawley rats into plastic syringes containing 0.1 vol of 3.6 % citrate anticoagulant. PRP was obtained by centrifuging (220 x g) blood for 8-10 min at 22 °C. The PRP was diluted 1:1 with calcium-free Tyrode solution (pH 6.5) containing 0.5 μg prostaglandin I_2/ml, 0.7 mM creatine phosphate, 2.0 U creatine phosphokinase/ml and 2 mM phosphorylcholine. Diluted PRP was centrifuged (900 x g) for 10 min to sediment platelets. The platelet pellet was re-suspended in fresh solution and used for labelling platelets.

B. Preparation of Labelled Platelets

1. Labelling with [^{3}H]Arachidonic Acid

Platelets suspended in calcium-free Tyrode solution (pH 6.5) containing 0.5 μg prostaglandin E_1/ml, instead of prostaglandin I_2, 0.7 mM creatine phosphate, and 2.0 U creatine phosphokinase/ml, were incubated with [^{3}H]arachidonic acid (7.5 μCi/ml) for 90 min as described by Nakano et al. (1987). Platelets were then washed (x3) by

centrifugation (900 x g) for 10 min in calcium-free Tyrode solution (pH 6.5) containing 0.5 μg prostaglandin E_1/ml, 0.7 mM creatine phosphate and 2.0 U creatine phosphokinase/ml, to remove the unincorporated radioactivity. Platelets were re-suspended in fresh solution by gentle agitation after each centrifugation step. The washed platelets were finally re-suspended in Tyrode-HEPES solution (pH 7.4) containing 0.25% (w/v) BSA and the platelet concentration was determined.

2. Labelling with [^{3}H]Inositol

Rat platelets were labelled with [^{3}H]inositol following a previously described procedure (Nakano et al., 1987). Briefly, platelets suspended in calcium-free Tyrode solution (pH 6.5) containing 0.5 μg prostaglandin E_1/ml, 0.7 mM creatine phosphate and 2.0 U creatine phosphokinase/ml were incubated with [^{3}H]inositol (50 μCi/ml) for 90 min at 37 °C. Platelets were subsequently washed as described in section X.B.1. The washed platelets were re-suspended in albumin-free Tyrode-HEPES solution (pH 7.4) and platelet concentration was determined.

3. Labelling with [^{3}H]Myristic Acid

Platelets were labelled with [^{3}H]myristic acid following the procedure similar to that used by Huang and Cabot (1990) to provided a PC pool specifically enriched by [^{3}H]myristic acid in fibroblasts. Platelet phospholipids were labelled by incubating platelets with [^{3}H]myristic acid (33 μCi/ml) for 90 min at 37 °C in calcium-free

Tyrode solution (pH 6.5) containing 0.5 μg prostaglandin E_1/ml, 0.7 mM creatine phosphate, and 2.0 U creatine phosphokinase/ml. Platelets were washed as described in section X.B.1. The washed platelets were finally re-suspended in Tyrode-HEPES solution (pH 7.4) containing 0.25% (w/v) BSA and the platelet concentration was determined.

To characterize the incorporation of [^{3}H]myristic acid into platelet lipids, the organic layer of extracted platelets (Bligh and Dyer, 1959) was evaporated to dryness, re-suspended in chloroform/methanol (1:1 v/v) and analyzed by TLC. This protocol usually resulted in incorporation of approximately 2,400,000 DPM/10^9 platelets.

4. Labelling with [^{14}C-*methyl*]Lyso PAF

The procedure used to label the choline phospholipid pool with either 1-O-[^{3}H]alkyl-LPC or 1-O-alkyl-[^{32}P]LPC in human platelets (Huang et al., 1991) was used to label rat platelets. Briefly, platelets were incubated with [N-*methyl*-^{14}C]lyso-PAF (2.5 μCi/ml) for 90 min at 37 ℃ in calcium-free Tyrode solution (pH 6.5) containing 0.5 μg prostaglandin E_1/ml, 0.7 mM creatine phosphate, and 2.0 U creatine phosphokinase/ml. Platelets were washed as described in section X.B.1. The washed platelets were finally re-suspended in Tyrode-HEPES solution (pH 7.4) containing 0.25% (w/v) BSA and the platelet concentration was determined.

C. Assay for PLA_2

To measure PLA_2 activity in intact platelets, [^{3}H]arachidonic acid-labelled platelets (5 x 10^8/ml) suspended in Tyrode-HEPES solution (pH 7.4) were incubated at 37 °C for 15 min with 1.5 mM $CaCl_2$. Platelets were then stimulated by thrombin in a platelet aggregometer with constant stirring and the reaction was allowed to occur for 5 min. In some cases platelets were pre-incubated in the presence of rat CRP (0.48 μM) and calcium for 15 min at 37 °C before adding thrombin. The 1 ml assay was finally stopped by adding 0.15 ml of 100 mM EDTA at pH 5.0 and cooling on ice. The reaction mixtures were extracted (Bligh and Dyer, 1959). The final organic layer removed from extracted samples was evaporated to dryness under a stream of N_2 and re-suspended in 150 μl of chloroform/methanol (1/1, v/v). Radioactive arachidonic acid metabolites or arachidonate-containing phospholipid in the organic extract were then separated by TLC.

D. Assay for PI-PLC

[^{3}H]inositol-labelled platelets (5 x 10^8/ml) in albumin-free Tyrode-HEPES solution (pH 7.4) containing 1.5 mM $CaCl_2$ and 10 mM LiCl were equilibrated in the absence or presence of rat CRP (0.48 μM) for 15 min. Platelets were then stimulated for 3 min by thrombin (0.05 U/ml or 2 U/ml) in a platelet aggregometer with constant stirring. The reaction was stopped by addition of 3.75 vol of chloroform/methanol (1/2, v/v) containing 1 N HCl and was extracted (Bligh and

Dyer, 1959). The aqueous layer was analyzed by ion-exchange chromatography (Dowex AG1-X8 formate form) for [^{3}H]inositol phosphates following the procedure of Berridge et al. (1983) with some modification. Briefly, the aqueous layer containing the water soluble inositol metabolites was neutralized with KOH (2 M) and diluted with 2 vol of distilled water. This solution was applied to a 1 ml column of AG1-X8 Dowex (formate form) ion exchange resin which had been equilibrated in 6 bed volumes of distilled water. [^{3}H]Inositol and glycerol-[^{3}H]inositol were eluted together in 5 ml of 60 mM ammonium formate/5 mM disodium formate. A second washing step consisting of 2 washings with 2.5 ml of 0.2 M ammonium formate/0.1 M formic acid eluted [^{3}H]inositol-1-phosphate. A third washing phase consisting of 2 washings with 2.5 ml of 0.4 M ammonium formate/0.1 M formic acid eluted [^{3}H]inositol-1,4-biphosphate while a fourth wash consisting of 2 washings of 2.5 ml of 1.0 M ammonium formate/0.1 M formic acid eluted [^{3}H]IP_3. The formation of [^{3}H]inositol-1-phosphate, [^{3}H]inositol-1,4-biphosphate and [^{3}H]IP_3 was measured by counting the fractions eluted from the column.

E. Assay for PC-PLC

1. Using Platelets Labelled with [^{3}H]Myristic Acid

Washed [^{3}H]myristic acid-labelled platelets (5 x 10^8/ml) suspended in Tyrode-HEPES solution (pH 7.4) were allowed to equilibrate for 15 min before use. The labelled platelets (1 ml) were then equilibrated in presence of 1.5 mM $CaCl_2$ and

presence or absence of rat CRP (0.48 μM) for 15 min at 37 °C before adding thrombin. After equilibration, [^{3}H]myristic acid-labelled platelets were stirred in a platelet aggregometer and were then stimulated for 3 min by thrombin (0.05 U/ml or 2 U/ml). The reaction was stopped by addition of 3.75 vol of chloroform/methanol (1/2, v/v) containing 2% acetic acid and extracted (Bligh and Dyer, 1959). The organic layer was evaporated to dryness under N_2 and the lipid residue was re-suspended in 100 μl of chloroform/methanol (1/1, v/v). Aliquots (50 μl) of the re-suspended lipid were analyzed by TLC to separate [^{3}H]DAG from other lipids. The radioactivity associated with DAG was expressed as a percentage of total radioactive lipid.

2. Using Platelets Labelled with [N-*methyl*-^{14}C]Lyso PAF

The [N-*methyl*-^{14}C]lyso-PAF-labelled platelets (5 x 10^8/ml) were finally re-suspended in Tyrode-HEPES solution (pH 7.4) and allowed to equilibrate for at least 15 min at 37 °C. Platelets were incubated in presence of 1.5 mM $CaCl_2$ and presence or absence of rat CRP (0.48 μM) for 15 min before stimulation. Stirred [N-*methyl*-^{14}C]lyso-PAF-labelled platelets were stimulated for 3 min by thrombin (0.05 U/ml or 2 U/ml). The reaction was stopped by addition of 3.75 vol of chloroform/methanol (1/2, v/v) containing 2% acetic acid and extracted (Bligh and Dyer, 1959). Aliquots of both the aqueous and organic layers of extracted samples were counted for radioactivity. An aliquot of the equilibrated platelet suspension was also stopped

immediately to act as a zero time control (to determine the background levels of radioactive products). The radioactivity associated with [^{14}C]phosphorylcholine that formed during each experiment was expressed as a percentage of total radioactivity after subtraction of zero time control. Identification of the aqueous soluble product as [^{14}C]phosphorylcholine was made by analysis of a 400 μl aliquot of the aqueous layers by TLC.

CHAPTER 5

STUDIES ON THE EFFECT OF RAT CRP ON PLATELET AGGREGATION

Section I. Introduction

While the precise physiological function of CRP remains to be established, its participation in various reactions, *in vitro*, may be of some relevance to its physiological function. Previous studies have shown an inhibitory effect of rabbit and human CRP on platelet aggregation induced by a number of agonists; however, the effect of rat CRP on platelet aggregation has not been investigated. The present study was, therefore, carried out to determine if rat CRP has an inhibitory effect on ADP-, thrombin- and PAF-induced platelet aggregation.

The refractory property of rat platelets to PAF has been reported in the literature (Cargill et al., 1983; Sanchez-Crespo et al., 1981; Vargiftig et al., 1981). One explanation put forward for this resistance to PAF may be the absence of high affinity PAF-binding sites on rat platelets (Inarrea et al., 1984). However, in view of the well known property of rat CRP to bind to the phosphorylcholine ligand, which is a part of the PAF molecule (Fig. 1), an alternate explanation may be that rat CRP, by interacting with PAF through the phosphorylcholine moiety, may inhibit the physiological action of PAF.

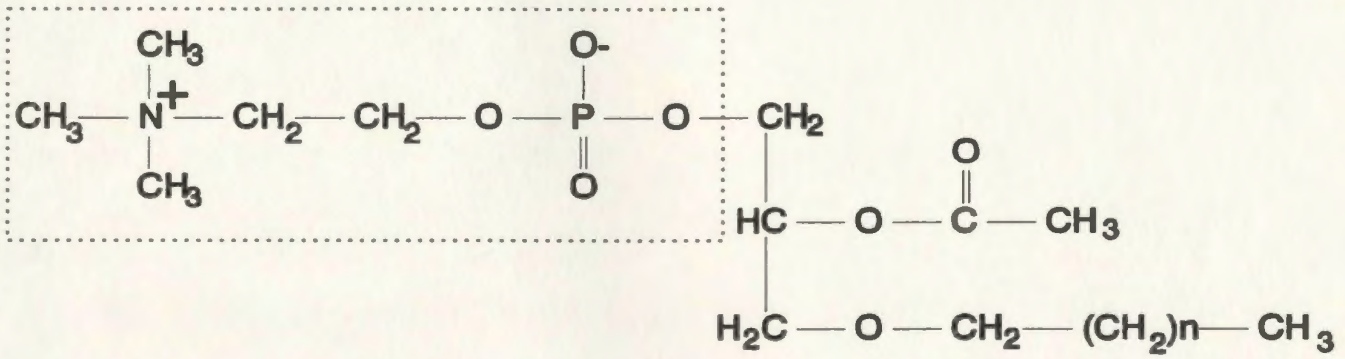


Fig. 1. **Structure of PAF and possible binding site for rat CRP.** 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. The alkyl chain is typically 16-18 carbons in length. Boxed is the phosphorylcholine moiety believed to be the binding site for rat CRP.

Section II. Results

A. PAF-Induced Aggregation of Rat Platelets

The results presented in Fig. 2 (bottom trace) confirms the previously reported observation of the refractory property of rat platelets to PAF-induced aggregation. Rat platelets in PRP did not aggregate in the presence of 0.5 μM PAF, which is a concentration of PAF that would normally result in maximum aggregation of human or rabbit platelets. However, when 25 μl of the rabbit antiserum to rat CRP was pre-incubated for 5 min with rat PRP, there was a marked increase in the aggregation of platelets by PAF (Fig. 2, top trace). A control experiment in which non-immune rabbit serum was pre-incubated with rat PRP did not show any PAF-induced platelet aggregation (result not shown).

Additional experiments to establish a role of rat CRP in the resistance of rat platelets to PAF were carried out using washed and unwashed rat platelets. Washed rat platelets were prepared using a 2 mM phosphorylcholine solution to remove any rat CRP bound to the platelet surface. When PAF (0.5 μM) was added to the platelets that had not been washed in phosphorylcholine-containing solution there was very little aggregation of the platelets (Fig. 3, bottom trace). In contrast, platelets that had been washed by the phosphorylcholine-containing solution showed a marked increase in aggregation by PAF (Fig. 3, top trace). Furthermore, when rat CRP (0.25 μM) was pre-incubated with washed platelets, platelets were once again shown to be resistant to PAF-induced aggregation (Fig. 4, bottom trace) compared to platelets in

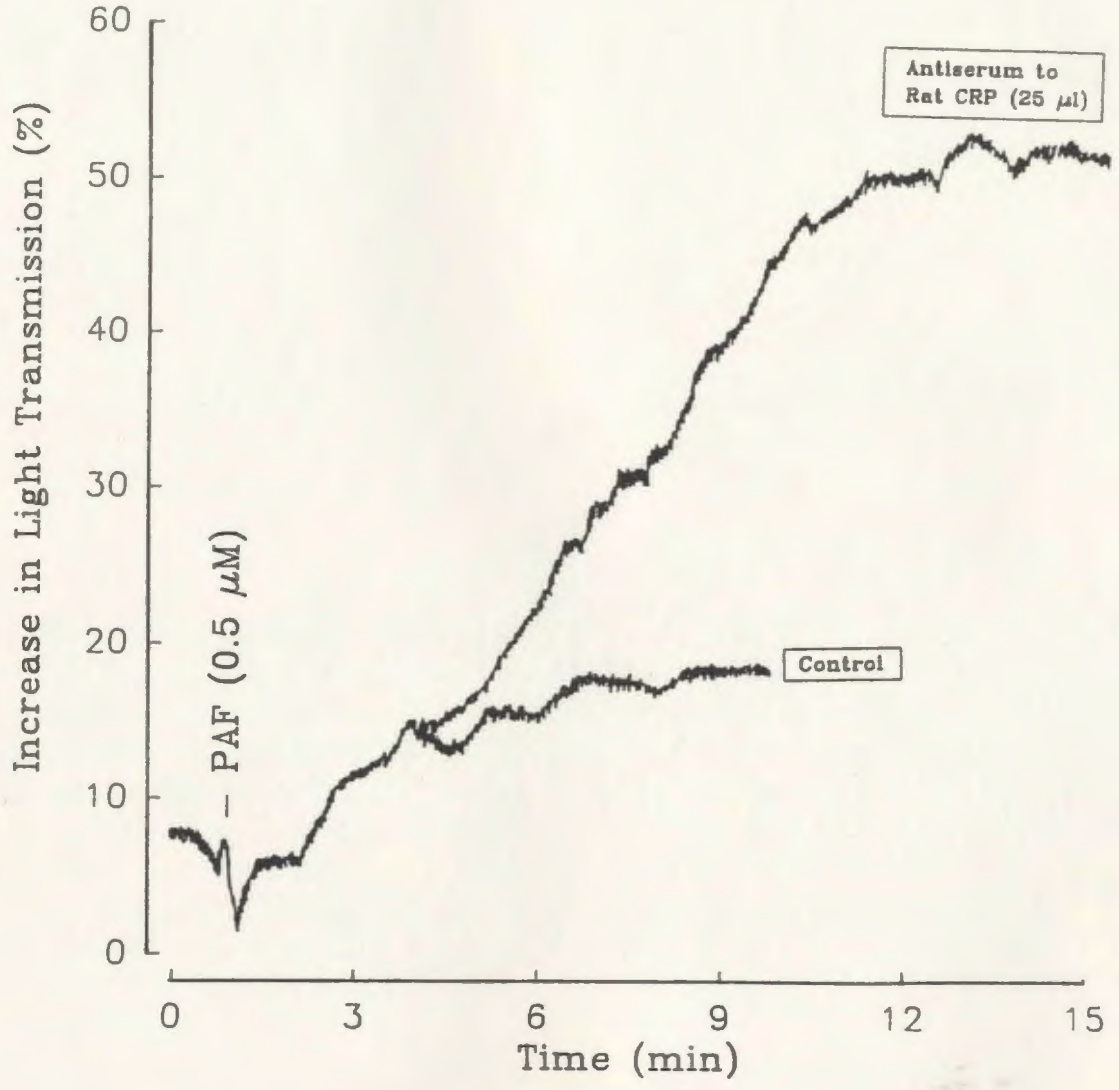


Fig. 2. **Effect of incubation of rabbit antiserum to rat CRP with rat PRP on PAF-induced aggregation of rat platelets.** PAF (0.5 μM) was added to rat PRP ($1.7x10^8$ in 450 μl) in the absence of the antiserum to rat CRP and the aggregation was recorded (Bottom trace). PRP was pre-incubated with antiserum to rat CRP (25 μl) for 5 min (Top trace). At the end of the incubation PAF (0.5 μM) was added and the platelet aggregation was recorded. Other experimental conditions were as described in Methods and Materials (section V.B.3). Results are typical of 4 experiments.

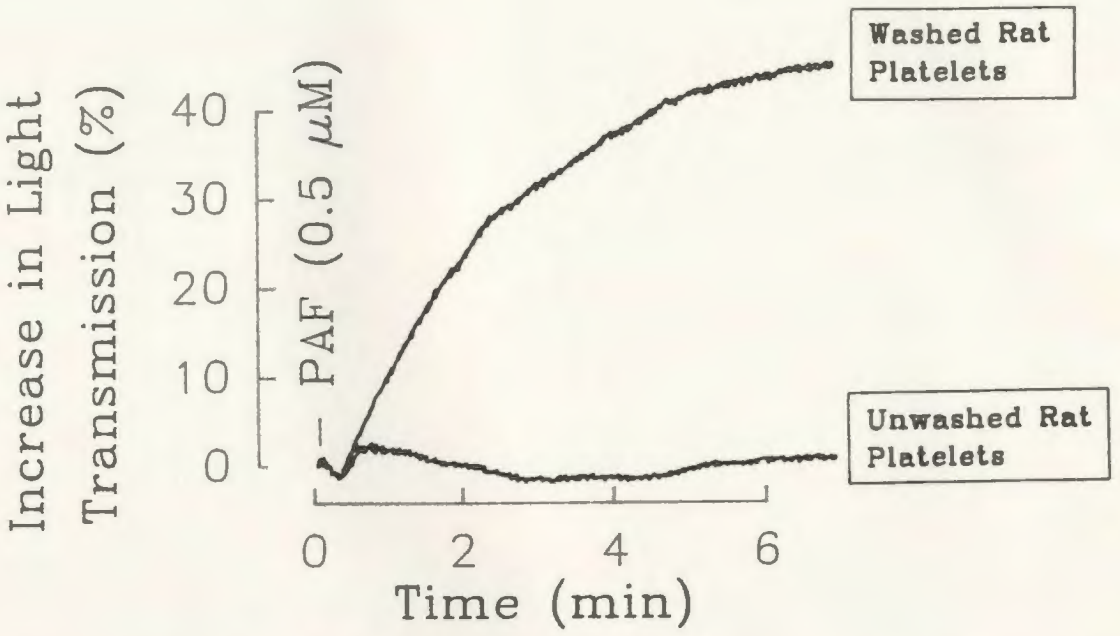


Fig. 3. **Effect of PAF on the aggregation of washed and unwashed rat platelets.** (Bottom trace) PAF (0.5 μM) was added to unwashed rat platelets (1.7 x 10^8 in 450 μl) that had been isolated from plasma by centrifugation (900 x g) for 10 min and re-suspended in 16 mM NaH_2PO_4 (pH 7.4) buffer containing 124 mM NaCl, 5 mM KCl, 4 mM glucose, and 1.5 mM $CaCl_2$. The aggregation was recorded after adding PAF to the platelets. (Top trace) PAF (0.5 μM) was added to rat platelets which were washed (x 2) in buffer containing 2 mM phosphorylcholine and (x 2) in buffer without phosphorylcholine. The washed platelets were re-suspended in 16 mM NaH_2PO_4 (pH 7.4) buffer containing 124 mM NaCl, 5 mM KCl, 4 mM glucose, and 1.5 mM $CaCl_2$, and the aggregation was recorded after adding PAF. Results are typical of 2 experiments.

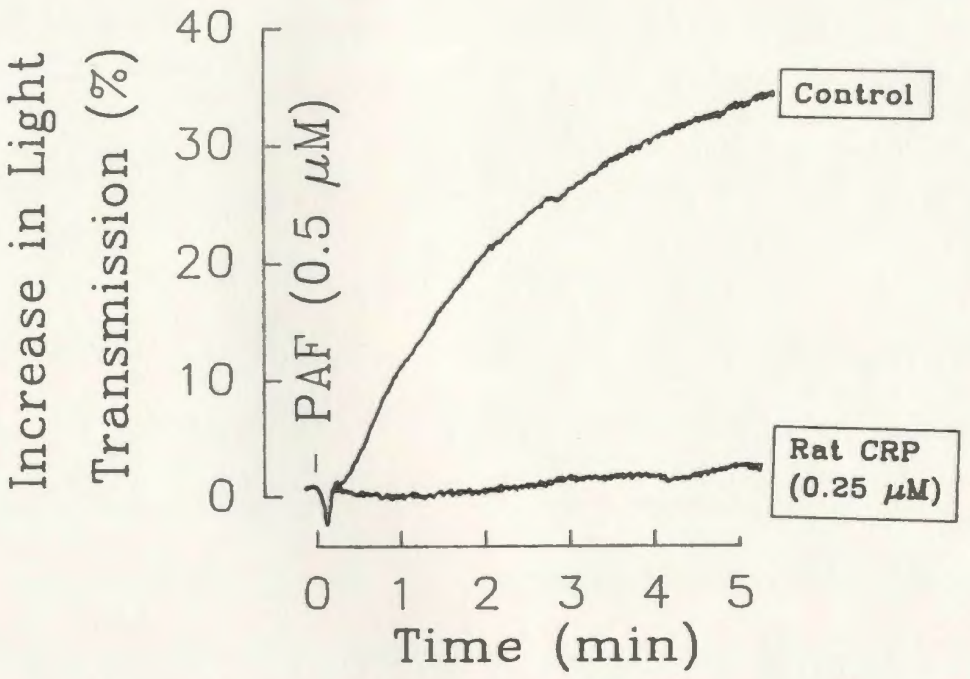


Fig. 4. **Effect of incubation of rat CRP with washed rat platelets on PAF-induced platelet aggregation.** (Top trace) PAF (0.5 μM) was added to washed rat platelets (1.7×10^8 in 450 μl) suspended in 16 mM NaH_2PO_4 (pH 7.4) buffer containing 124 mM NaCl, 5 mM KCl, 4 mM glucose, and 1.5 mM $CaCl_2$, and the aggregation was recorded. (Bottom trace) PAF (0.5 μM) was added to washed rat platelets which had been pre-incubated with rat CRP (0.25 μM) for 5 min and the aggregation was recorded. Results are typical of 2 experiments.

the absence of rat CRP (Fig. 4, top trace).

B. Effect of Rat CRP on PAF-Induced Platelet Aggregation

The property of rat CRP to inhibit PAF-induced platelet aggregation was also studied using human PRP and washed rabbit platelets. The platelets from these species are known to aggregate in the presence of PAF. Because CRP is not normally present in rabbit and human plasma, the platelets from these species were not expected to possess any surface-bound CRP. Therefore, the addition of PAF to human PRP and rabbit platelets resulted in platelet aggregation (shown in Figs. 5 and 6). In the presence of increasing concentrations of rat CRP (0-0.27 μM) there was a dose-dependent inhibition of PAF-induced aggregation of human PRP (Fig. 5A). In this experiment rat CRP was pre-incubated with PRP before the addition of PAF (0.5 μM). In another experiment rat CRP (5 μM) was pre-incubated with PAF (0.5 μM) which was then added to the PRP (Fig. 5B). Experiments following either protocol resulted in the inhibition of PAF-induced platelet aggregation by rat CRP. In experiments using washed rabbit platelets, rat CRP also inhibited PAF-induced platelet aggregation (Fig. 6)

C. Binding of Rat CRP to [^{3}H-alkyl]PAF

[^{3}H-alkyl]PAF (0.05 nmol) in 50 mM Na_2SO_4, 20 mM NaH_2PO_4 buffer (pH 6.8) containing 2.5 mM $CaCl_2$ was applied to a TSK-Sperogel (7.5 X 300 mm;

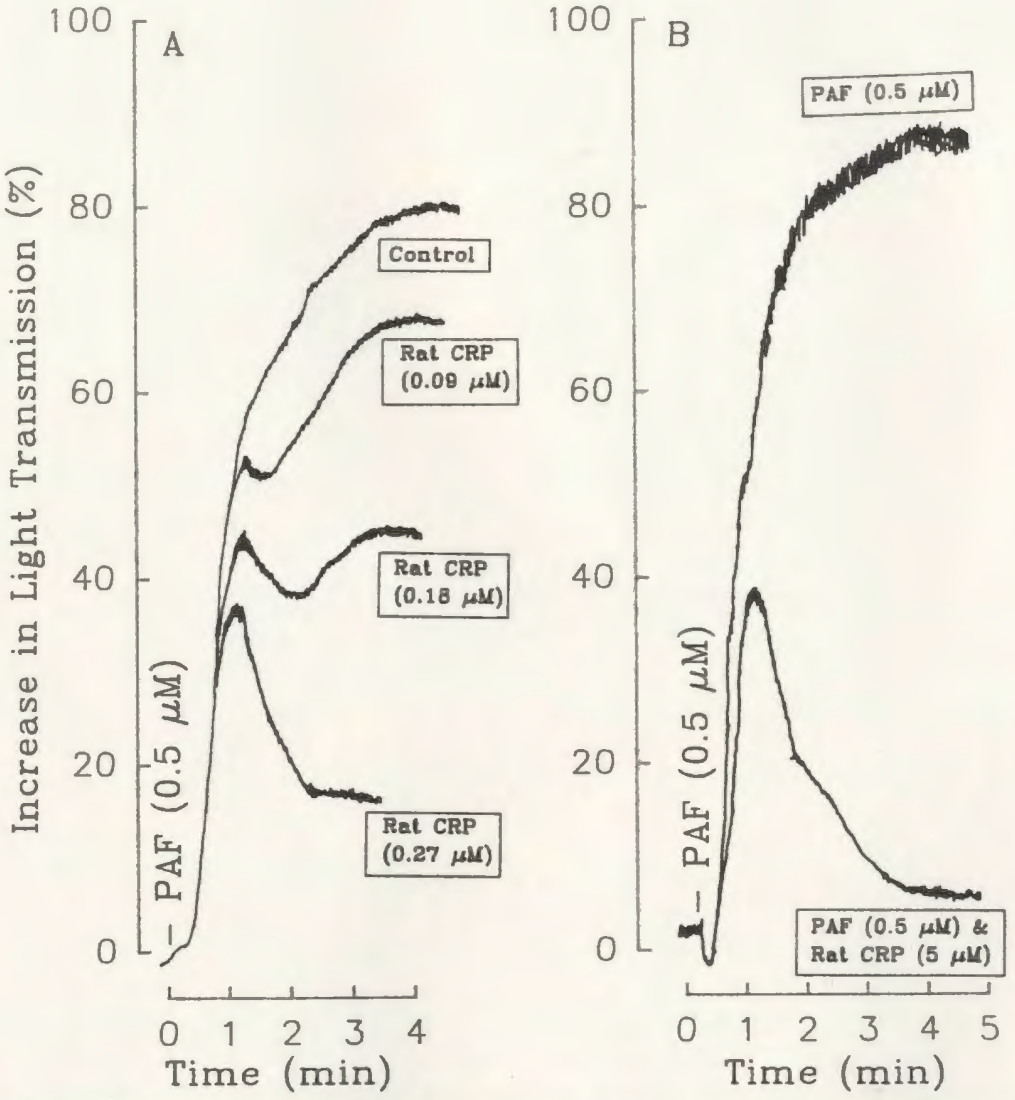


Fig. 5. **Effect of rat CRP on PAF-induced aggregation of human platelets.** (A) Increasing amounts of rat CRP (0-0.27μM) were pre-incubated for 5 min with human PRP (1.7 x 10^8 platelets in 450 μl). At the end of the incubation period PAF (0.5 μM) was added and the aggregation was recorded. The upper most trace (control) shows the result of incubation in absence of rat CRP. (B) Rat CRP (5 μM) was also pre-incubated with PAF (0.5 μM) for 10 min and then added to human PRP (bottom trace), and aggregation was recorded. Aggregation induced by addition of PAF (0.5 μM) alone to human PRP is shown in the upper trace (control). Results are typical of 2 experiments.

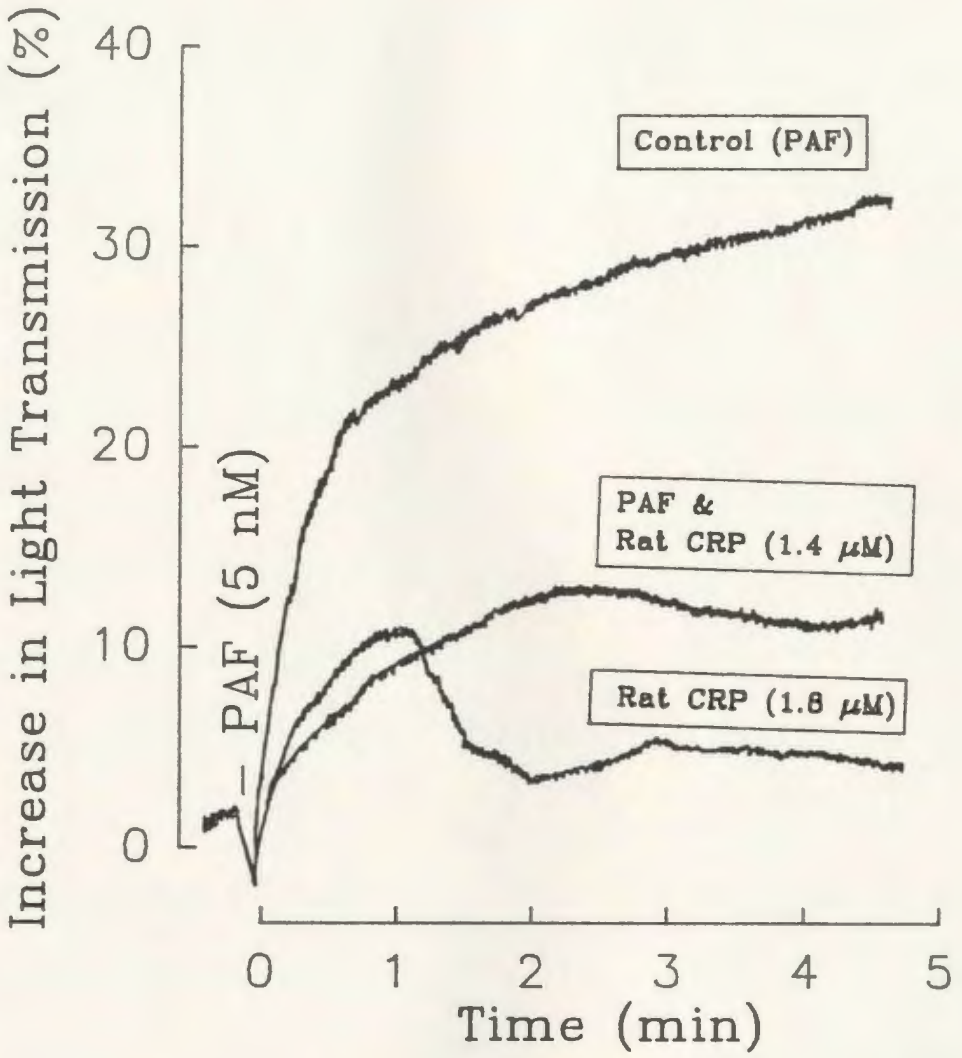
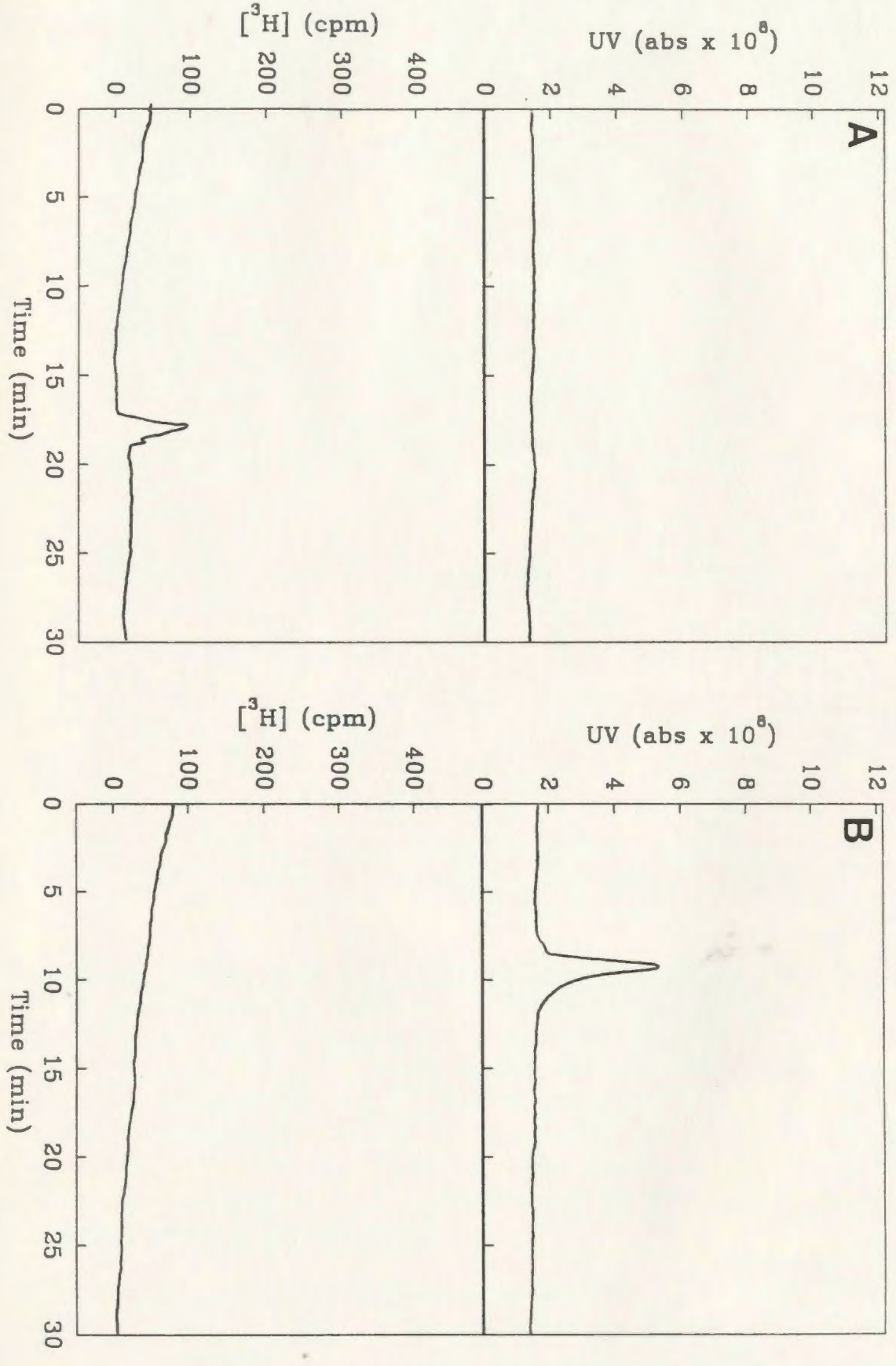


Fig. 6. **Effect of rat CRP on PAF-induced aggregation of washed rabbit platelets.** (Top trace; control) PAF (5 nM) was added to washed rabbit platelets (1.7×10^8 platelets in 450 μl) suspended in Tyrode solution containing 1 mM $CaCl_2$ and aggregation was recorded. (Middle trace) Rat CRP (1.4 μM) was pre-incubated for 10 min with PAF (5 nM). At the end of the incubation period the mixture was added to the washed rabbit platelets and the aggregation was recorded. (Bottom trace) PAF (5 nM) was added to washed rabbit platelets that had been pre-incubated for 10 min with rat CRP (1.8 μM) and aggregation was recorded. Results are typical of 3 experiments.

Beckman) HPLC gel-filtration column maintained at a flow rate of 0.8 ml/min and connected to a radioisotope detector. The [^{3}H-alkyl]PAF eluted from the column at 18 min (Fig. 7A, bottom trace). No corresponding absorbance peak was detected using a UV detector (Fig. 7A, top trace). Under identical conditions the elution of rat CRP from the HPLC gel filtration column occurred at 9.5 min (Fig. 7B, top trace). No corresponding radioactive peak was detected by the radioisotope detector (Fig. 7B, bottom trace). The formation of a rat CRP-[^{3}H-alkyl]PAF complex occurred on incubation of 0.5 nmol rat CRP with 0.05 nmol [^{3}H-alkyl]PAF and was observed by chromatography on HPLC gel filtration column (Fig. 8). Most of the rat CRP bound [^{3}H-alkyl]PAF eluted with a higher molecular weight species, possibly an aggregated complex of native rat CRP. A minor peak with an elution time similar to that of native rat CRP was also observed. This complex formation did not occur in the absence of calcium (Fig. 9) and was inhibited by the pre-incubation of rat CRP with phosphorylcholine in the rat CRP-[^{3}H-alkyl]PAF incubation mixture (Fig. 10). Inhibition of the rat CRP-[^{3}H-alkyl]PAF complex formation by phosphorylcholine was dose-dependent (result not shown). The binding of rat CRP to [^{3}H-alkyl]PAF could also be inhibited by excess unlabelled PAF (result not shown). The binding of [^{3}H-alkyl]PAF to albumin and α_1-acid glycoprotein, which was previously reported (Matsumoto and Miwa, 1985; McNamara et al., 1986), is shown in Fig. 11. The PAF complex formed with these proteins was not affected by phosphorylcholine or calcium.

Fig. 7. **HPLC elution profile of rat CRP and [^{3}H-alkyl]PAF.** A) [^{3}H-alkyl]PAF (0.05 nmol) in a final volume of 50 μl and absence of rat CRP was applied to a HPLC gel filtration column. B) Rat CRP (0.5 nmol) in a final volume of 50 μl and absence of [^{3}H-alkyl]PAF was applied to a HPLC gel filtration column. The upper trace represents the absorbance elution profile detected at 280 nm by LC-95 UV/Visible Spectrophotometer Detector. The lower trace is the radioactivity trace as detected by a Beckman 171 Radioisotope Detector with windows set for tritium as described in Methods and Materials (section VII.A.).


A
B
UV (abs x 10^6)
[3H] (cpm)
Time (min)
0
2
4
6
8
10
12
100
200
300
400
5
15
20
25
30

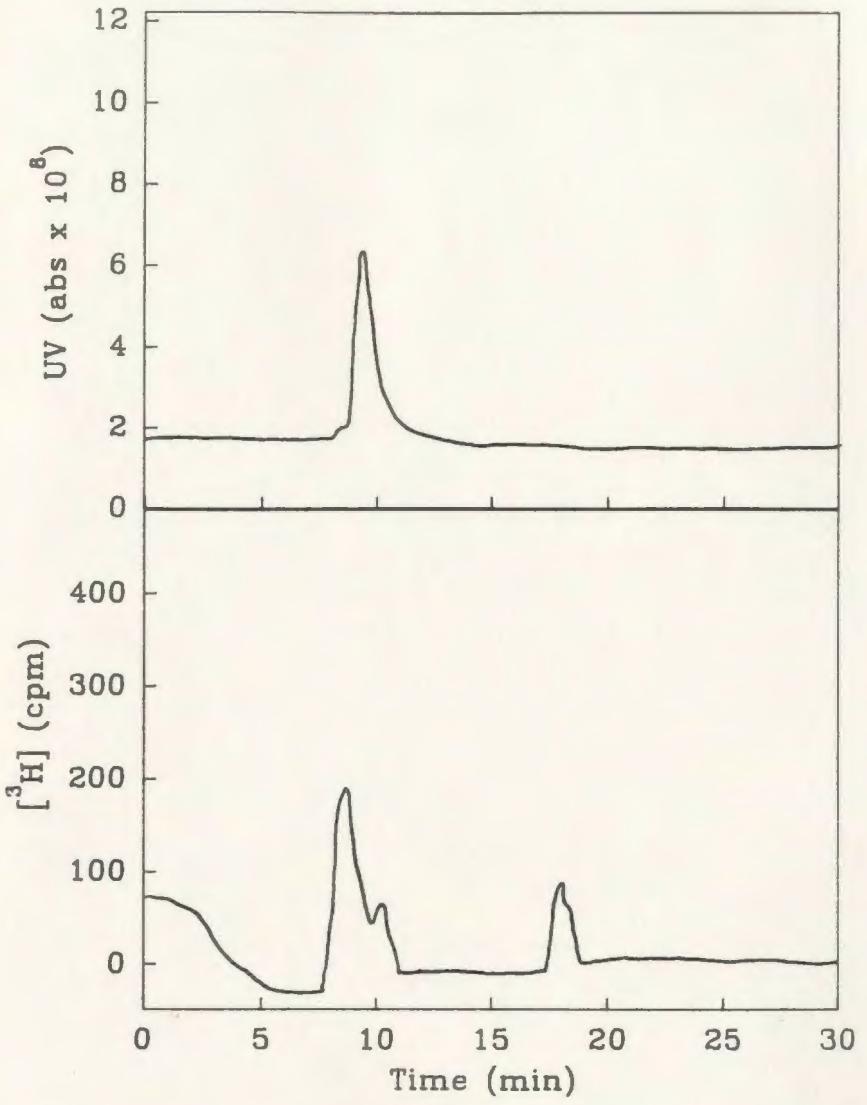


Fig. 8. **HPLC elution profile of rat CRP-[^{3}H-alkyl]PAF complex.** Rat CRP (0.5 nmol) was pre-incubated for 10 min with [^{3}H-alkyl]PAF (0.05 nmol) in a final volume of 50 μl. The mixture was then applied to a HPLC gel filtration column (TSK-Sperogel; (7.5 mm x 300 mm)) equilibrated with 2.5 mM $CaCl_2$, 50 mM Na_2SO_4, 20 mM NaH_2PO_4 buffer (pH 6.8) at a flow rate of 0.8 ml/min. The upper trace represents the absorbance elution profile detected at 280 nm by LC-95 UV/Visible Spectrophotometer Detector. The lower trace is the radioactivity trace as detected by a Beckman 171 Radioisotope Detector with windows set for tritium as described in Methods and Materials (section VII.A.).

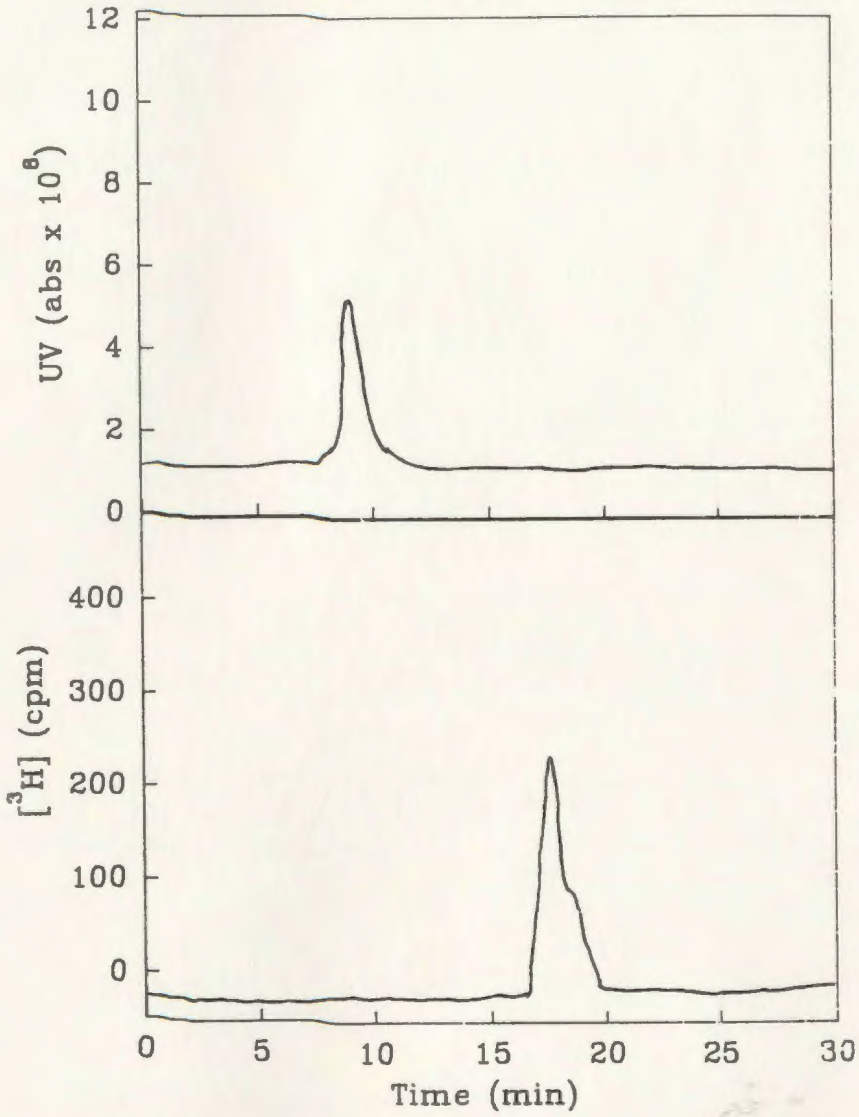


Fig. 9. **Effect of absence of calcium on formation of the rat CRP-[^{3}H-alkyl]PAF complex.** Rat CRP (0.5 nmol) was pre-incubated for 10 min with [^{3}H-alkyl]PAF (0.05 nmol) in absence of calcium in a final volume of 50 μl. The mixture was then applied to a HPLC gel filtration column equilibrated with 50 mM Na_2SO_4, 20 mM NaH_2PO_4 buffer (pH 6.8). The upper and lower traces are as described in Fig. 8.

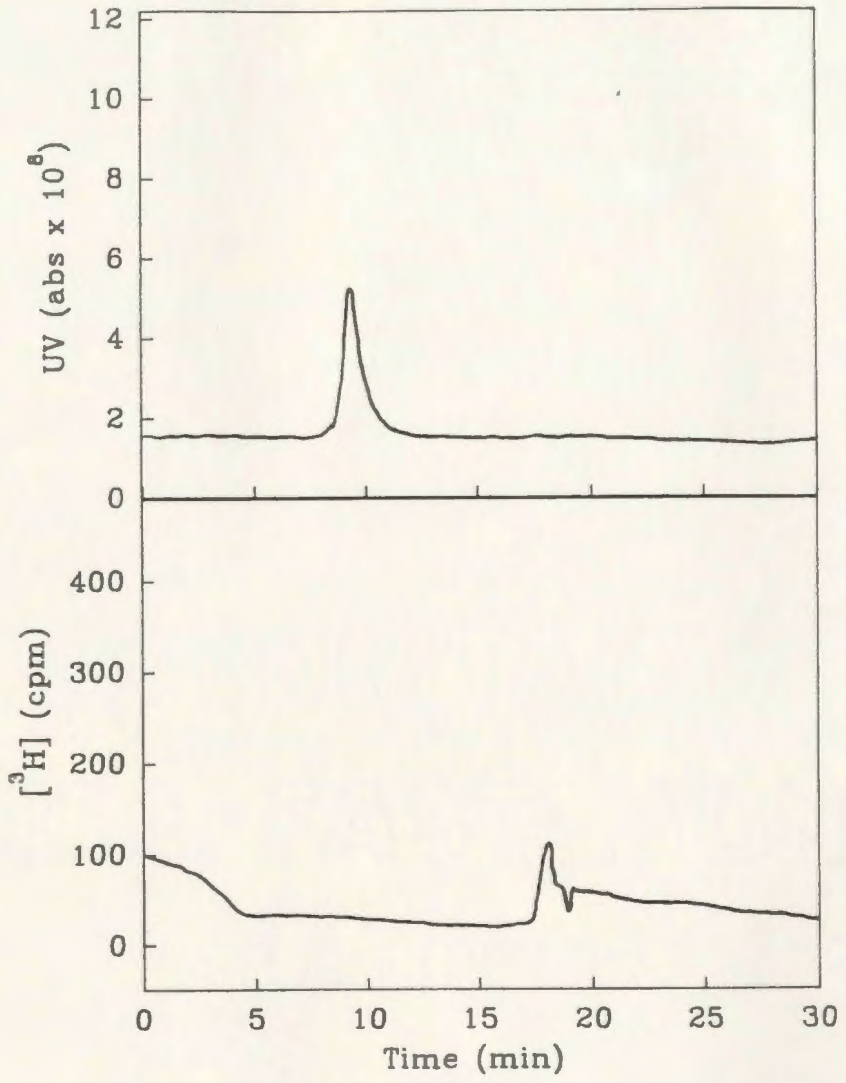
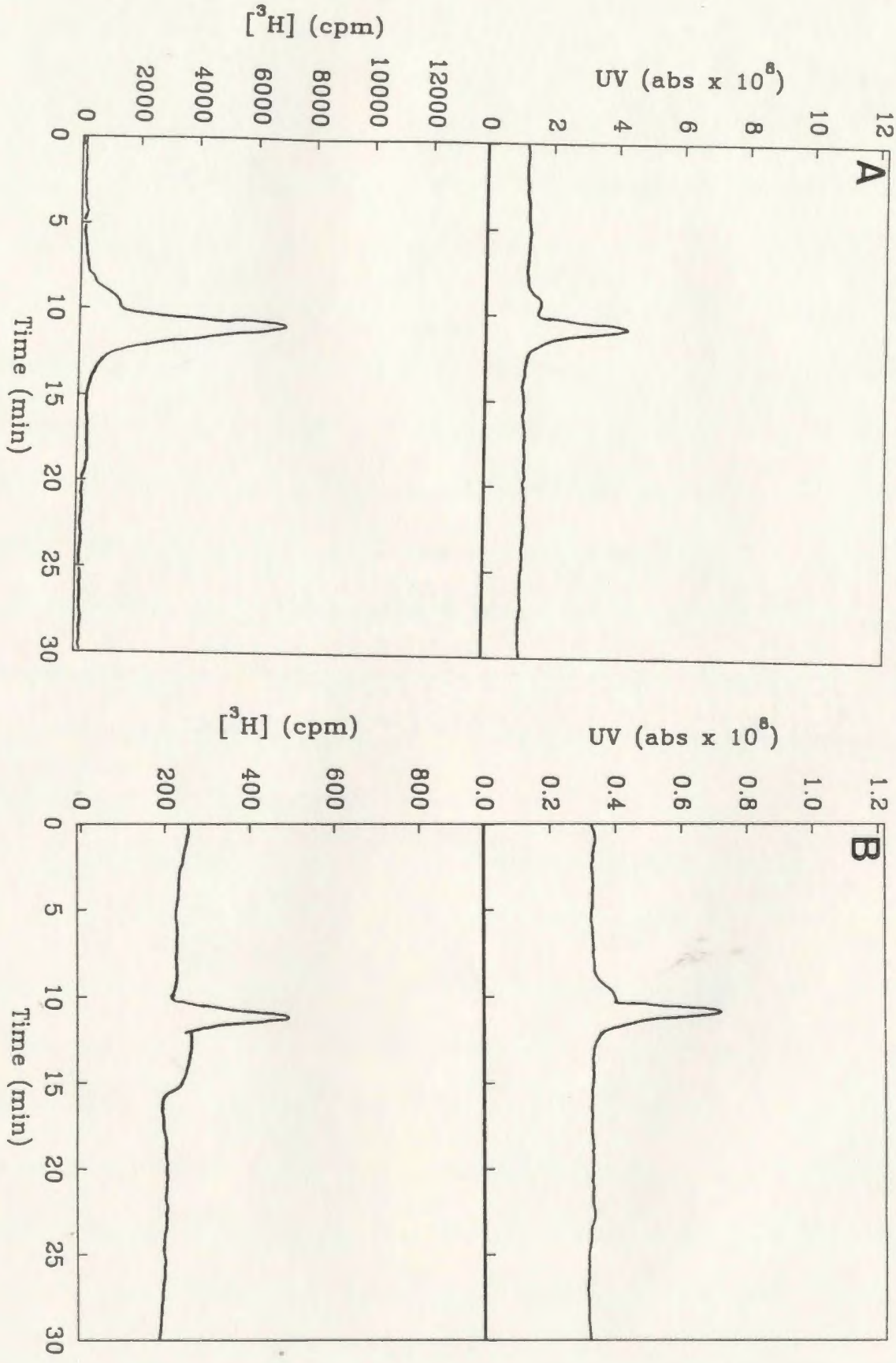


Fig. 10. **Effect of phosphorylcholine on formation of the rat CRP-[^{3}H-alkyl]PAF complex.** Rat CRP (0.5 nmol) in presence of phosphorylcholine (0.5 μmol) was pre-incubated for 10 min with [^{3}H-alkyl]PAF (0.05 nmol) in a final volume of 50 μl. Other experimental conditions were as described for Fig. 8.

Fig. 11. **HPLC elution profiles of albumin-[^{3}H-alkyl]PAF complex and α_1-acid glycoprotein-[^{3}H-alkyl]PAF complex.** A) Albumin (0.5 nmol) was pre-incubated for 10 min with [^{3}H-alkyl]PAF (0.05 nmol) in a final volume of 50 μl and then applied to a HPLC gel filtration column. B) α_1-Acid glycoprotein (0.5 nmol) was pre-incubated for 10 min with [^{3}H-alkyl]PAF (0.05 nmol) in a final volume of 50 μl and applied to a HPLC gel filtration column. The upper and lower traces are as described for Fig. 8.


A
UV (abs x 10^6)
[3H] (cpm)
Time (min)
B
UV (abs x 10^6)
[3H] (cpm)
Time (min)

D. Effect of Rat CRP on ADP- and Thrombin-Induced Aggregation of Rat Platelets

To determine if the inhibitory effect of rat CRP on platelet aggregation was specific to PAF, the effect of rat CRP on platelet aggregation induced by other agonists was examined. The addition of ADP (44 μM) to washed rat platelets resulted in platelet aggregation (Fig. 12). When increasing concentrations of rat CRP (0-0.36 μM) were pre-incubated with the platelets, there was a dose-dependent inhibition of ADP-induced platelet aggregation. In contrast, when rat CRP was pre-incubated with ADP and then added to the platelets, there was no inhibition of platelet aggregation (result not shown). The inhibition of platelet aggregation by rat CRP was also examined in the presence of phosphorylcholine (0.31-31 μM). The result in Fig. 13 shows a dose-dependent inhibition of the effect of rat CRP on ADP-induced platelet aggregation by phosphorylcholine.

To determine whether the observed inhibition of platelet aggregation by rat CRP was specific to rat CRP, the effect of other serum proteins on ADP-induced platelet aggregation was examined. Fig. 14 shows that the property to inhibit platelet aggregation was specific to rat CRP (0.36 μM) and α_1-acid glycoprotein (1 μM) since ovalbumin (0.71 μM) and fetuin (0.71 μM) did not inhibit the ADP-induced platelet aggregation. In contrast, α_1-acid glycoprotein (1 μM) inhibited the ADP-induced platelet aggregation. The inhibition of ADP-induced platelet aggregation of human platelets by α_1-acid glycoprotein was previously reported (Costello et al., 1979).

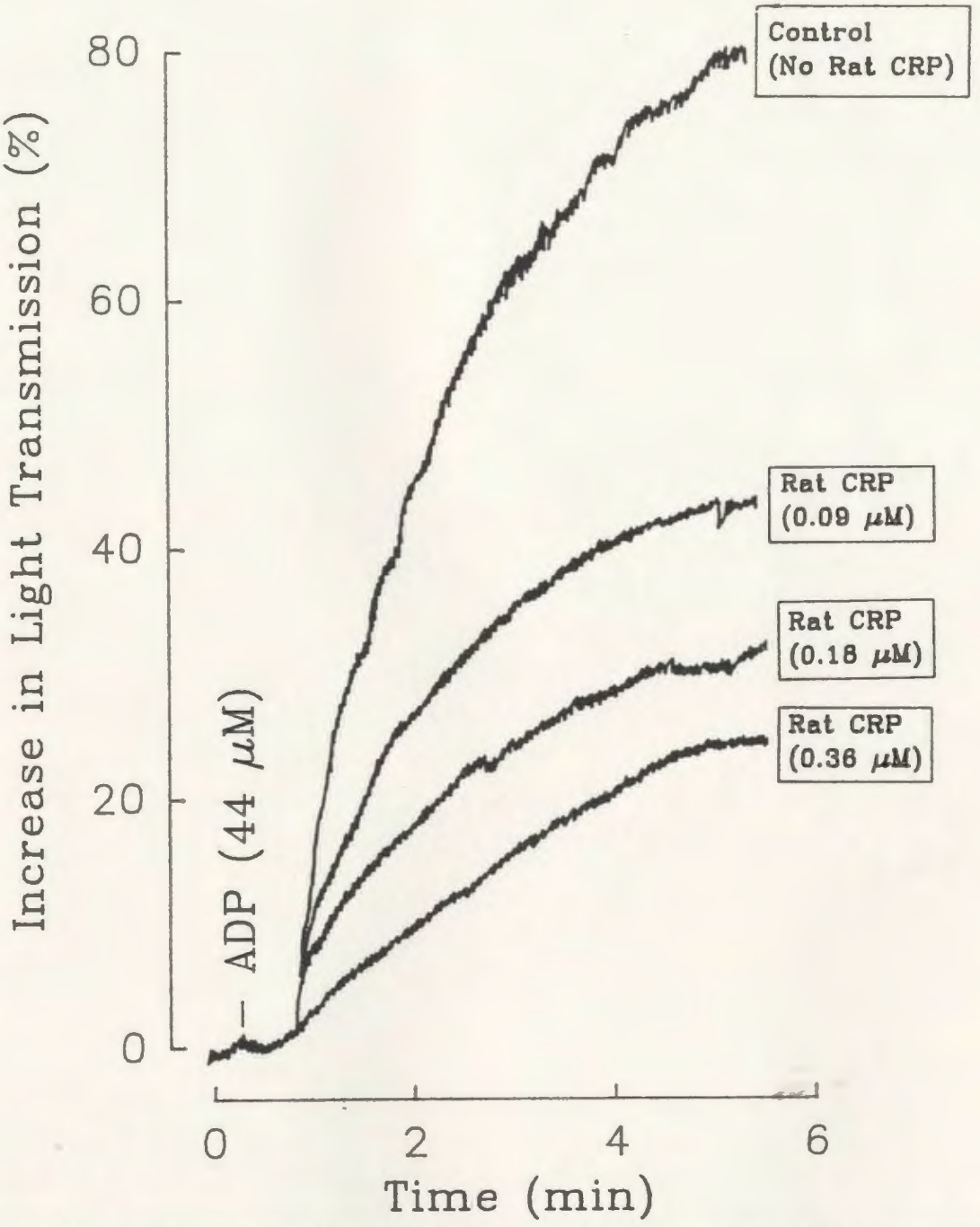


Fig. 12. **Effect of rat CRP on ADP-induced aggregation of washed rat platelets.** Washed rat platelets (1.7×10^8 in 450 μl) were pre-incubated for 5 min with varying amounts of rat CRP (0-0.36 μM). At the end of the 5 min incubation, ADP (44 μM) was added to each incubation and the platelet aggregation was recorded. The upper most trace is the control which refers to ADP-induced aggregation of washed rat platelets in the absence of rat CRP. Results are typical of 4 experiments.

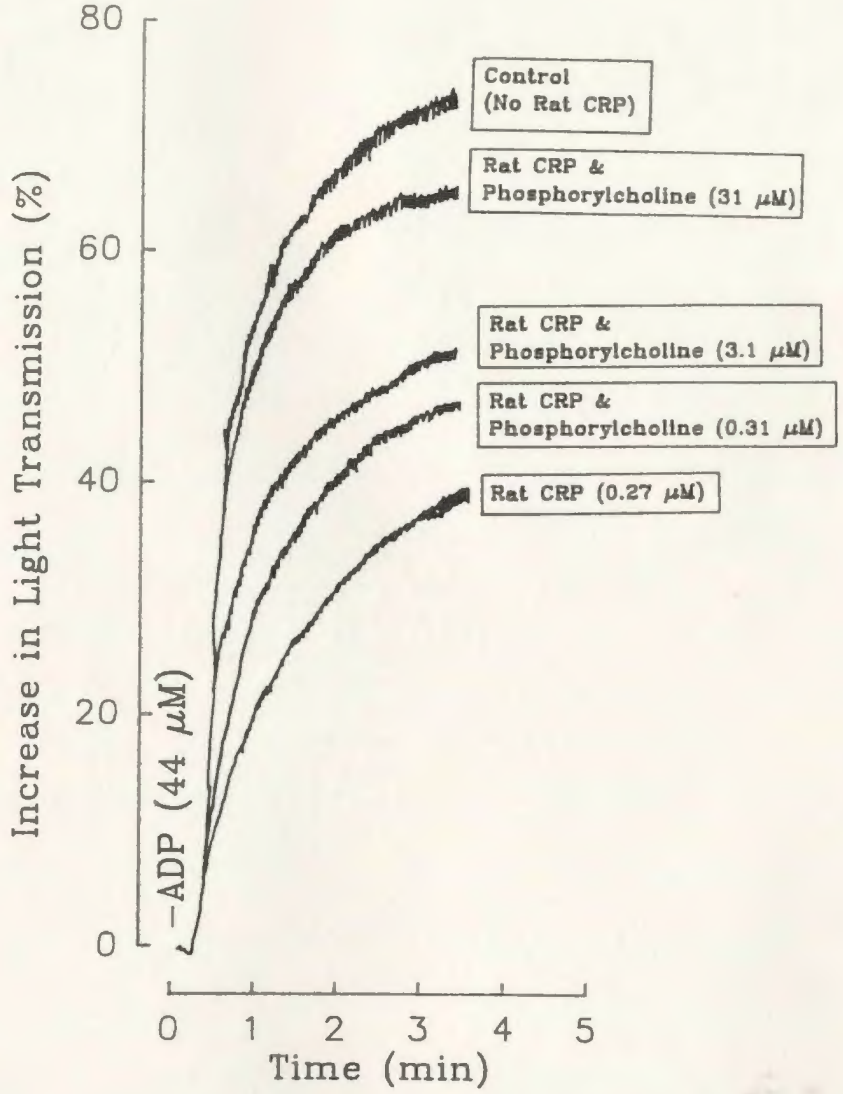


Fig. 13. **Reversal of the inhibition of ADP-induced rat platelet aggregation by phosphorylcholine.** Rat CRP (0.27 μM) which was pre-incubated for 1 min in presence or absence of increasing concentrations of phosphorylcholine (0.31-31.0 μM). This mixture was added to washed rat platelets (1.7×10^8 in 450 μl) and allowed to incubate for another 5 min, after which time ADP (44 μM) was added and the aggregation was recorded. Control refers to ADP-induced aggregation of washed rat platelets in the absence of rat CRP. Results are typical of 2 experiments.

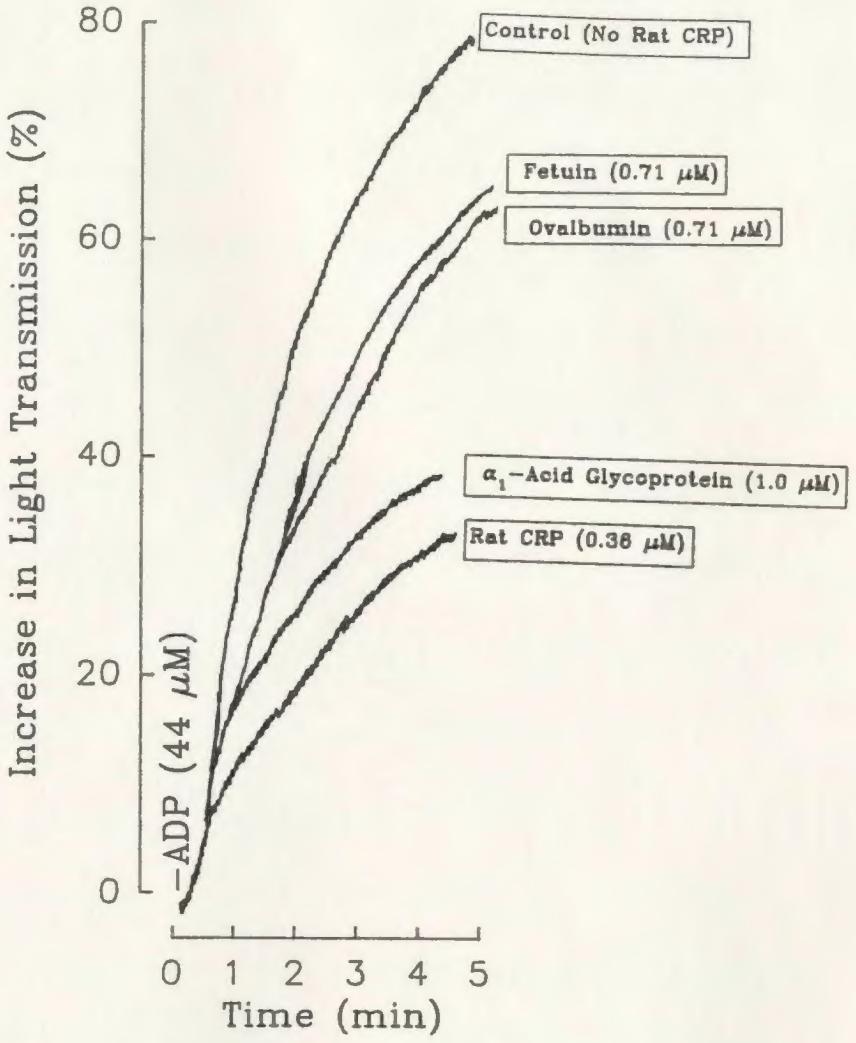


Fig. 14. **Effect of certain glycoproteins on the ADP-induced aggregation of washed rat platelets.** Washed rat platelets (1.7×10^8 in 450 μl) were incubated for 5 min with either fetuin (0.71 μM), ovalbumin (0.71 μM), α_1-acid glycoprotein (1.0 μM) or rat CRP (0.36 μM). After the incubation platelet aggregation was initiated by addition of ADP (44 μM). In the control experiment the platelets were incubated for 5 min in the absence of rat CRP or other proteins, and the platelets were aggregated with ADP (44 μM). Results are typical of 2 experiments.

In addition to studying the effect of rat CRP on PAF and ADP-induced platelet aggregation, the effect of rat CRP on thrombin-induced platelet aggregation was also examined. The inhibitory effect of rat CRP on platelet aggregation induced by 0.1 U thrombin/ml, was dependent on the concentration of rat CRP pre-incubated with the platelets (Fig. 15). The maximal inhibitory effect of rat CRP on thrombin-induced platelet aggregation was observed when 0.48 μM rat CRP was pre-incubated with the platelets. Higher concentrations of rat CRP (up to 1.6 μM) did not increase the inhibitory effect. Studies carried out in which rat CRP was pre-incubated with thrombin before the addition to platelets had no effect on thrombin-induced platelet aggregation (result not shown).

Section III. Discussion

Although, PAF is a very potent inducer of platelet aggregation in many species (Vargiftig et al., 1981), rat platelets are refractory to PAF-induced platelet aggregation and are not stimulated beyond shape change (Cargill et al., 1983). One explanation for this anomalous behaviour of rat platelets is that the resistance of rat platelets to PAF-induced platelet aggregation could be due to the absence of specific PAF-receptors on rat platelets (Inarrea et al., 1984). However, when rabbit antiserum to rat CRP was incubated with rat platelets the results showed a very pronounced PAF-induced aggregation of platelets (Fig. 2). This suggests that the refractory property of rat PRP to PAF-induced platelet aggregation was due to the

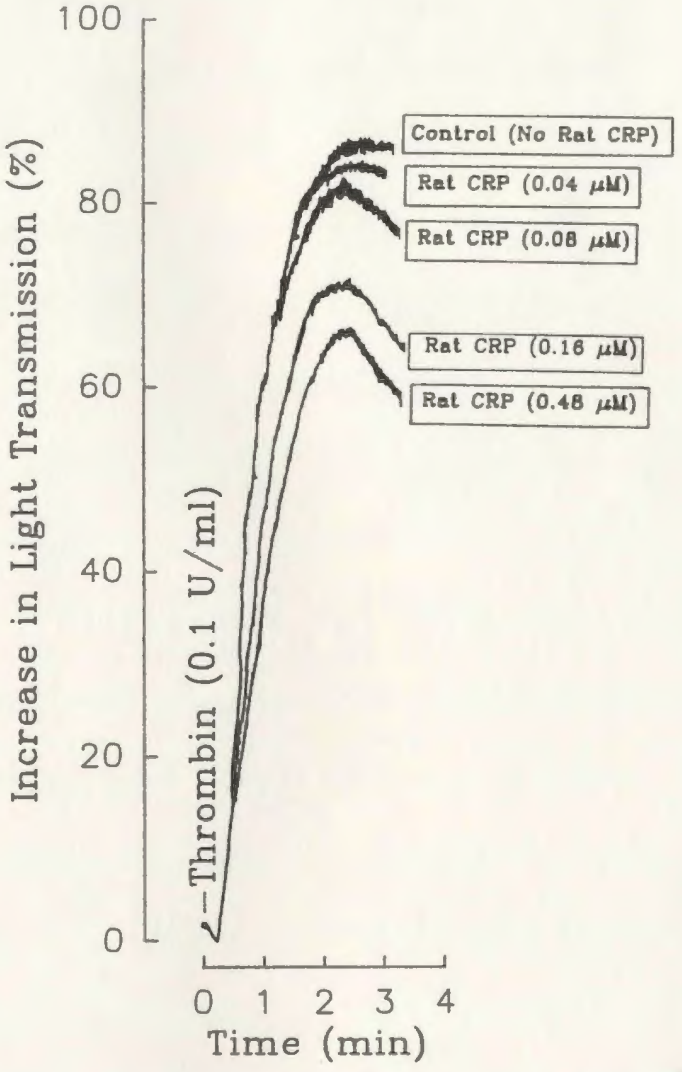


Fig. 15. **Effect of increasing concentrations of rat CRP on thrombin-induced platelet aggregation.** Washed rat platelets (4 x 10^8/ml), suspended in Tyrode solution (pH 7.4), were pre-incubated in presence of increasing concentrations of rat CRP (0-0.48 μM) for 15 min at 37 °C. Platelet aggregation was measured after adding thrombin (0.1 U/ml) to the suspension. In the control experiment (top trace), rat platelets were pre-incubated in the absence of rat CRP and platelets were aggregated with thrombin (0.1 U/ml). Results are typical of 4 experiments.

presence of rat CRP in the PRP preparation. This conclusion was further substantiated by the results of experiments using unwashed rat platelets and platelets washed with a solution containing 2 mM phosphorylcholine (to remove platelet-bound CRP). When PAF was added to unwashed rat platelets there was very little PAF-induced platelet aggregation. In contrast, the platelets that were washed with a phosphorylcholine-containing solution aggregated by the addition of PAF to the suspension (Fig. 3). Furthermore, the PAF-induced aggregation of washed platelets was subsequently inhibited when rat CRP was added to the platelets (Fig. 4). The underlying conclusion, therefore, is that rat CRP was bound to the surface of unwashed platelets and that washing of the platelets with a solution containing phosphorylcholine resulted in the removal of surface-bound rat CRP. These observations provide convincing evidence that the presence of rat CRP in rat PRP is responsible for the inhibition of PAF-induced platelet aggregation. In support of this observation, a previous report has also suggested that the presence of certain plasma proteins may inhibit PAF-induced platelet activation (Lanara et al., 1982).

Rat CRP which is normally present in rat PRP could inhibit the PAF-induced platelet aggregation by two possible mechanisms: 1) by interacting with PAF, or 2) by interacting with the platelets. These possibilities were examined by incubating rat CRP with either platelets or PAF and examining the effect on platelet aggregation. The results showed that the incubation of rat CRP with either PAF or with platelets resulted in an inhibitory effect on PAF-induced platelet aggregation (Figs. 5 and 6).

This indicates that rat CRP could effectively inhibit PAF-mediated platelet aggregation by interacting with either platelets or with PAF. Experiments in which human or rabbit CRP was used to study PAF-induced platelet aggregation also produced results which showed that CRP could inhibit PAF-induced platelet aggregation by interacting with PAF or with platelets (Hokama et al., 1984; Vigo, 1985; Kilpatrick and Virella, 1985; Filep et al., 1991).

Evidence for an inhibitory effect of rat CRP on PAF-induced platelet aggregation, along with the presence of a phosphorylcholine moiety on the PAF molecule presented the possibility of a fluid-phase interaction between rat CRP and PAF. The extreme insolubility of PAF coupled with its high non-specific binding to glassware, made it impossible to demonstrate a rat CRP-PAF interaction using conventional techniques like equilibrium dialysis or equilibrium gel-filtration. However, the co-elution of [^{3}H-alkyl]PAF with rat CRP, using a HPLC gel-filtration technique, clearly indicates a fluid-phase rat CRP-PAF interaction. Unlike the binding of PAF to either albumin or α_1-acid glycoprotein which was independent of calcium, the rat CRP-PAF interaction was clearly dependent on the presence of calcium, since in its absence there was no co-elution of [^{3}H-alkyl]PAF with rat CRP. Results also clearly demonstrate the involvement of the phosphorylcholine-binding site of rat CRP and the corresponding involvement of the phosphorylcholine moiety of PAF on the rat CRP-PAF interaction. Although attempts were made to determine the stoichiometry of the binding of rat CRP to PAF, the results obtained were inconclusive and therefore

an estimate of the number of PAF molecules bound to rat CRP was not possible. However, based on the results of a previous study which showed that rat CRP has a capacity to bind to 3 molecules of phosphorylcholine (Rassouli et al., 1992), it is possible that this stoichiometric relationship may also be true for PAF.

The bulk of the protein-bound PAF eluted as a higher molecular weight species (8.5 min). This indicated that [^{3}H-alkyl]PAF bound to an aggregated form of rat CRP. A smaller peak, however, co-eluted with rat CRP (9.5 min) which corresponds to the molecular weight of native rat CRP (Fig. 8). In support of the existence of aggregated forms of rat CRP, it should be pointed out that human CRP and its analogues have been shown to aggregate by calcium (Baltz et al., 1982) or by polycations and in high temperature (Fiedel et al., 1982c and d; Fiedel, 1985).

A complex between PAF and CRP, a circulating serum protein (in humans during acute phase response), may have some potential significance to the regulation of the biological activity of PAF. The existence of rat CRP as an inhibitor of some of the very destructive biological effects of PAF may, therefore, be essential to the survival of the rat, especially during inflammation or allergic responses when PAF is produced in significant quantities.

Fiedel and co-workers were first to demonstrate that human CRP inhibits platelet aggregation stimulated by ADP as well as other agonists like epinephrine, collagen, poly-L-lysine, and thrombin (Fiedel and Gewurz, 1976a and b; Fiedel et al., 1977). Rat CRP, like human CRP, showed a dose-dependent inhibitory effect on the

ADP-induced aggregation of washed rat platelets (Fig. 12). The inhibitory action of rat CRP on ADP-induced platelet aggregation, may be attributed to the phosphorylcholine-binding site on rat CRP, since experiments carried out in the presence of phosphorylcholine showed a dose-dependent reversal of rat CRP-induced inhibition of platelet aggregation (Fig. 13). Fiedel et al. (1976) also reported the inhibition of platelet aggregation by a specific anti-phosphorylcholine myeloma protein (T-15 mouse IgA myeloma protein) was reversed by phosphorylcholine, indicating the importance of the phosphorylcholine-binding site in inhibition of platelet function. It is most likely that in the case of ADP-induced platelet aggregation, rat CRP could inhibit platelet aggregation by binding to the platelet PC, and not to ADP, as results obtained on pre-incubation of rat CRP with ADP showed no inhibitory effect on platelet aggregation.

The inhibitory effect on ADP-induced platelet aggregation was specific to rat CRP and α_1-acid glycoprotein. This conclusion was supported by results from experiments using two other glycoproteins, fetuin and ovalbumin (Fig. 14). It is interesting to note that α_1-acid glycoprotein also inhibited ADP-induced platelet aggregation, but the concentration for inhibition was much greater than that of rat CRP. The inhibitory effect of α_1-acid glycoprotein on ADP-induced platelet aggregation was also described in a previous study (Costello et al., 1979). It is possible that the acidic nature of the two proteins (α_1-acid glycoprotein pI 2.9-3.4; rat CRP pI 3.8) may in some way contribute to their inhibitory effect on platelet aggregation.

The effect of rat CRP on platelet aggregation was also examined using thrombin as an agonist. Thrombin is a strong agonist and is one of the most effective platelet stimuli. At low concentrations, thrombin behaves as a weak agonist and can induce platelet aggregation at concentrations as low as 0.04 U/ml (Lapetina, 1990; Greco and Jamieson, 1991). Rat CRP had a dose-dependent inhibitory effect on thrombin-induced platelet aggregation (Fig. 9). The platelet aggregation that resulted in response to thrombin was accompanied by dissociation of the platelet aggregates in the presence of rat CRP. This may suggest that the mechanisms responsible for formation of stable aggregates during platelet activation may also be inhibited by rat CRP.

In conclusion, the results indicate an inhibitory role of rat CRP in the regulation of platelet aggregation induced by PAF, ADP or thrombin. It is possible that either a rat CRP-platelet interaction or, in the case of PAF, a rat CRP-PAF interaction may be involved in the inhibitory effect of rat CRP on platelet aggregation. Based on these results, the biochemical basis of the rat CRP-platelet interaction was further examined and is reported in chapter 6.

CHAPTER 6

STUDIES ON THE INTERACTION OF RAT CRP WITH PLATELETS

Section I. Introduction

The results in Chapter 5 indicated the involvement of an interaction between rat CRP and platelets in the inhibition of platelet aggregation induced by PAF, ADP, or thrombin. In addition, the reversal of the inhibitory effect of rat CRP on platelet aggregation by phosphorylcholine, indicated the involvement of the phosphorylcholine-binding site on rat CRP in its interaction with the platelets. Therefore, in order to confirm the involvement of a rat CRP-platelet interaction in the inhibition of platelet aggregation, it was necessary to determine the nature of the interaction between rat CRP and platelets. Furthermore, there has been no published report that characterized the binding of rat CRP to platelets, although the binding of rat CRP to platelets during platelet-mediated cytoxicity against schistomaisis has been reported (Bout et al., 1986). In this chapter, the binding of rat CRP to rat, rabbit and human platelets has been studied and the nature of the binding has been characterized.

Section II. Results

A. Conditions for Binding of Rat [^{125}I]CRP to Platelets.

In order to demonstrate the binding of rat CRP to platelets a binding assay was developed which involved incubating rat platelets (4 x 10^7 platelets/ml) with rat [^{125}I]CRP at 37 °C in an incubation medium composed of 20 mM Tris-HCl buffer (pH 7.4) containing 0.25 % BSA (w/v), 0.15 M NaCl, and 5 mM $CaCl_2$. The binding assays were carried out in 1.5 ml Eppendorf microfuge tubes previously coated with 0.25% BSA solution. The number of platelets (4 x 10^7/ml) used in the binding assays was selected from the platelet dose curve (Fig. 1).

The binding of rat [^{125}I]CRP to platelets was dependent on the time of incubation and calcium concentration used. The time-dependent binding of rat CRP to rat platelets is shown in Fig. 2A. Maximum specific binding was observed at 30 min of incubation with no significant increase in binding after this time period. The calcium dependence of the binding of rat CRP to rat platelets is shown in Fig. 2B. Binding did not occur when calcium was absent from the incubation mixture. However, an extracelluar calcium concentration of about 5 mM was required for maximum binding.

B. Binding of Rat [^{125}I]CRP to Rat, Rabbit and Human Platelets.

The binding of rat CRP to rat, rabbit, and human platelets was examined

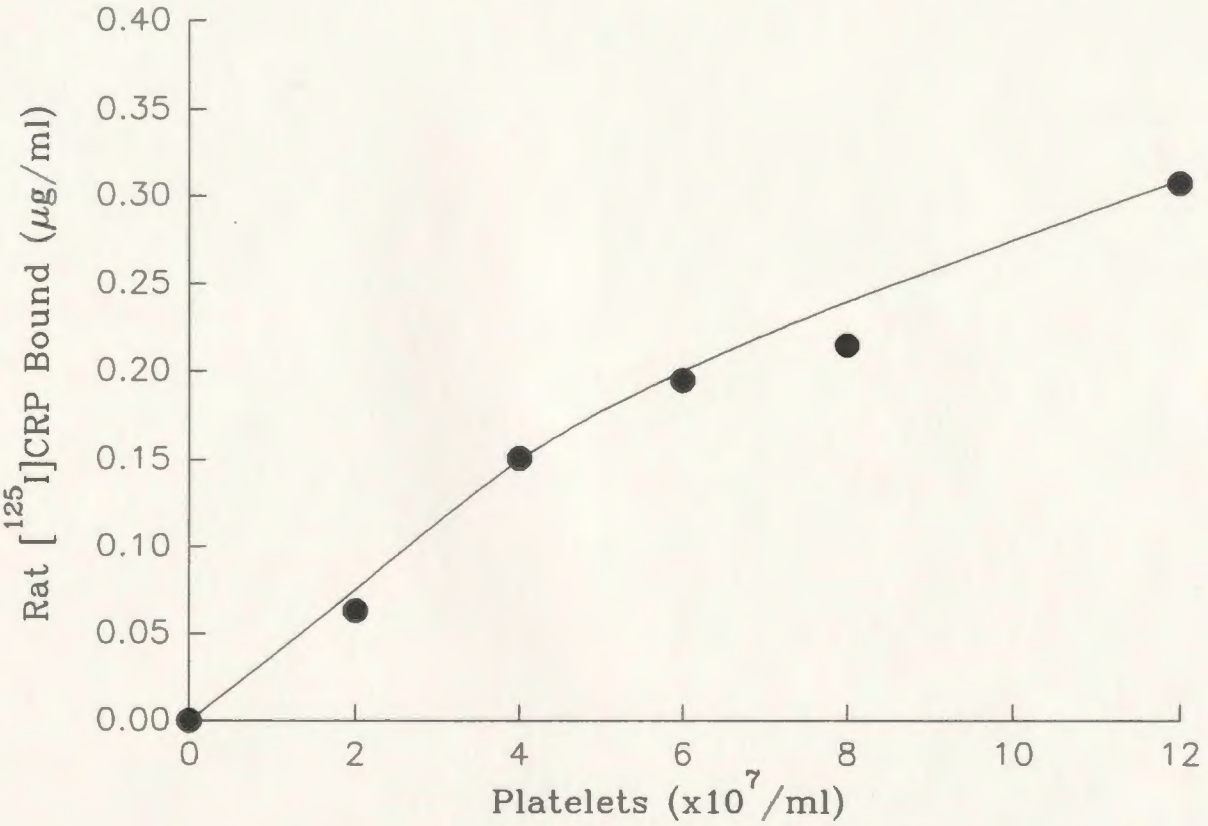
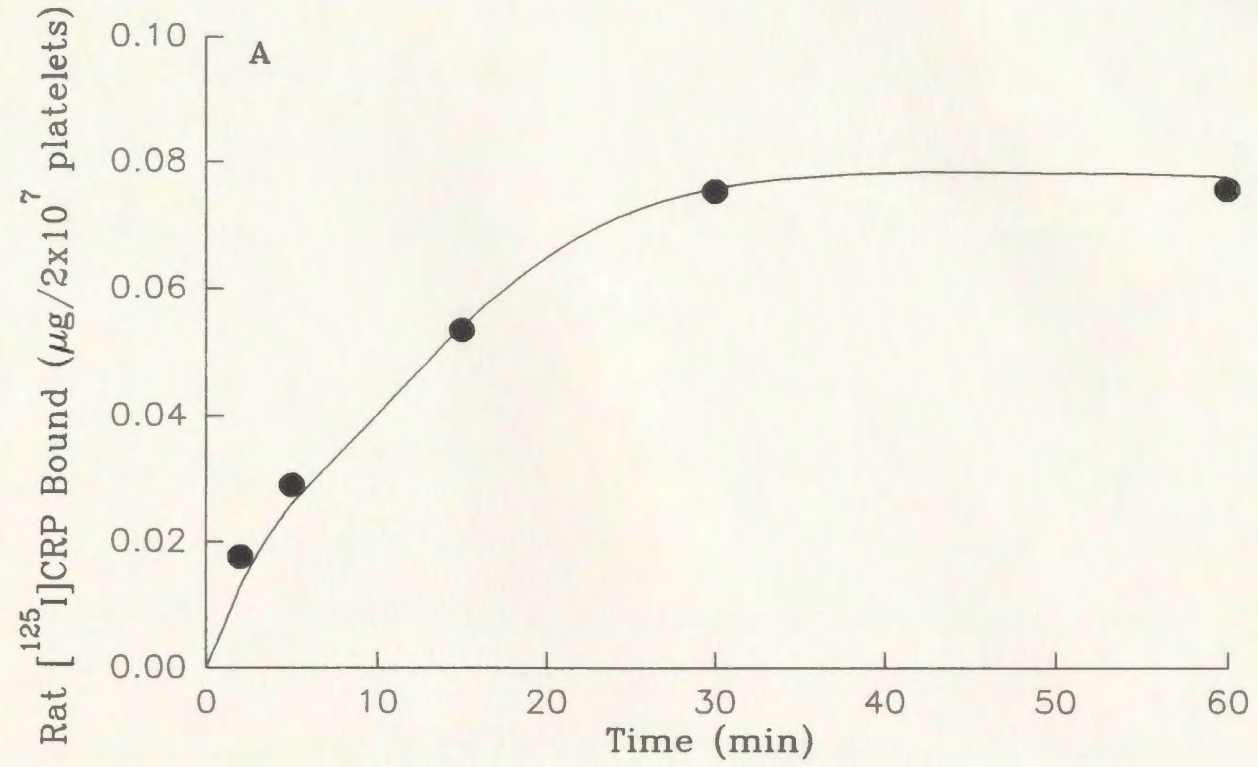
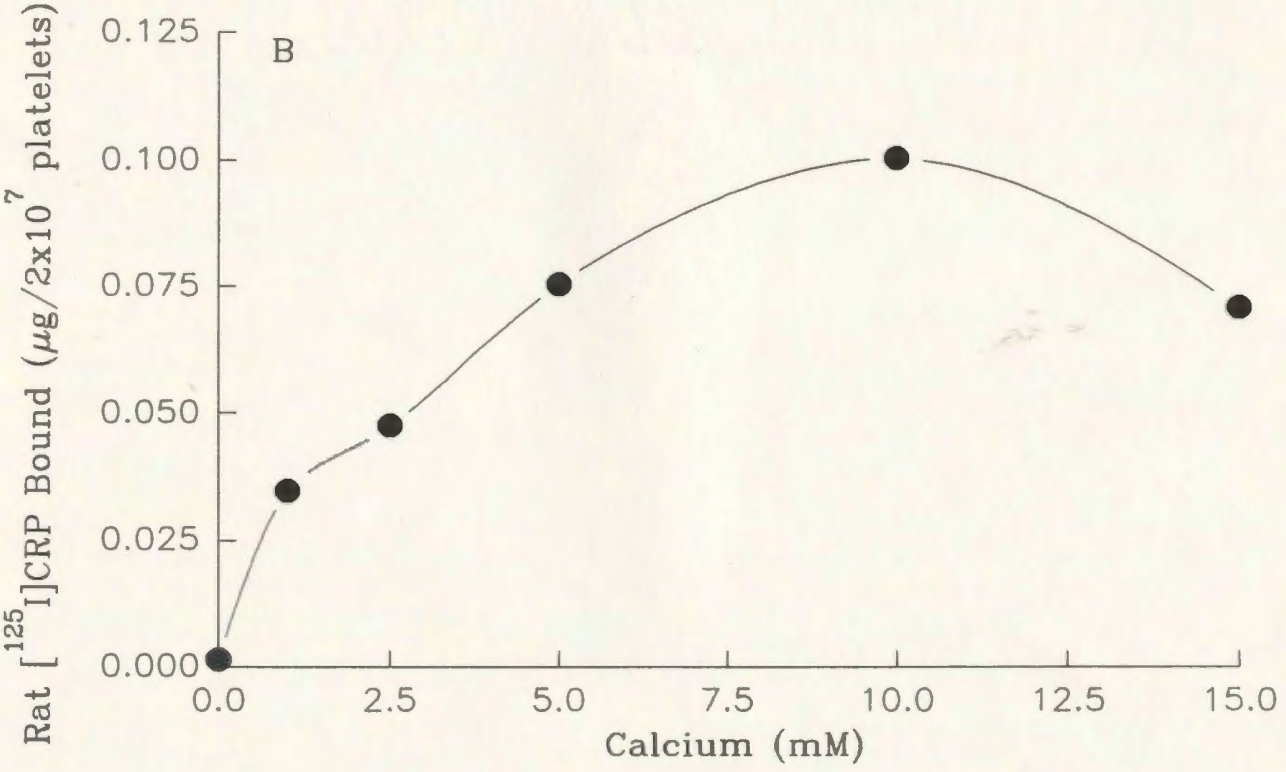


Fig. 1. **Binding of rat [^{125}I]CRP to rat platelets as a function of platelet concentration.** Binding studies were carried out with increasing concentrations of washed rat platelets (2 x 10^7-12 x 10^7/ml) and rat [^{125}I]CRP (2 μg/ml) for 30 min in 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.25% BSA (w/v). Assay was stopped by centrifuging at 12,800 x g for 2 min. The platelet pellet was washed (1 x) in ice cold buffer and radioactivity associated with it was counted by γ-counter. Non-specific binding was determined by adding 50-fold excess of unlabelled rat CRP. Data are mean of 2 experiments.

Fig. 2. **Binding of rat [^{125}I]CRP to rat platelets as a function of calcium concentration and time of incubation.** A) Time-dependent binding of rat [^{125}I]CRP to rat platelets. Rat [^{125}I]CRP (2 μg/ml) was added to washed rat platelets (4 x 10^7/ml) and incubated at 37 °C. Incubation was stopped at 1, 5, 15, 30, and 60 min. B) Calcium-dependence of the binding of rat CRP to rat platelets. Rat [^{125}I]CRP (2 μg/ml) was incubated with washed platelets (4 x 10^7/ml) in presence of increasing concentrations of $CaCl_2$ (0-15 μM) at 37 °C. Incubation was stopped at 30 min and the amount of rat [^{125}I]CRP bound to the platelets was determined as described in Fig. 1. Data are mean of 2 experiments.


A
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
Time (min)
Rat [125I]CRP Bound (μg/2x10^7 platelets)


B
0.125
0.100
0.075
0.050
0.025
0.000
0.0
2.5
5.0
7.5
10.0
12.5
15.0
Calcium (mM)
Rat [125I]CRP Bound (μg/2x10^7 platelets)

under optimum time and calcium concentration. The binding of rat [^{125}I]CRP to rat platelets was dose-dependent, saturable, and specific at 37 ℃ (Fig. 3). Similarly, the binding of rat CRP to rabbit and human platelets was also dose-dependent, saturable and specific (Fig. 4). Scatchard analysis of the binding data revealed the existence of a single class of high affinity CRP binding sites on rat, rabbit and human platelets (Figs. 3 and 4; Table 1). The rat, rabbit and human platelet binding capacity for rat [^{125}I]CRP ranged from 7.5 x 10^3 $\pm$ 1.9 x 10^3 sites/platelets for human platelets to 52 x 10^3 $\pm$ 15 x 10^3 sites/platelets for rabbit platelets. The dissociation constants (K_d) for

Table. 1. **Scatchard analysis parameters for binding of rat [^{125}I]CRP to platelets.** Statistics are mean $\pm$ S.D. for four separate experiments.

Source	Sites/Platelet	K_d
Rat	37 x 10^3 $\pm$ 10 x 10^3	45.2$\pm$14.9 nM
Rabbit	52 x 10^3 $\pm$ 15 x 10^3	26.1$\pm$8.3 nM
Human	7.5 x 10^3 $\pm$ 1.9 x10^{3*}	32.2$\pm$9.9 nM

*Binding curve plateau indicates approximately 5 x 10^3 sites/platelet.

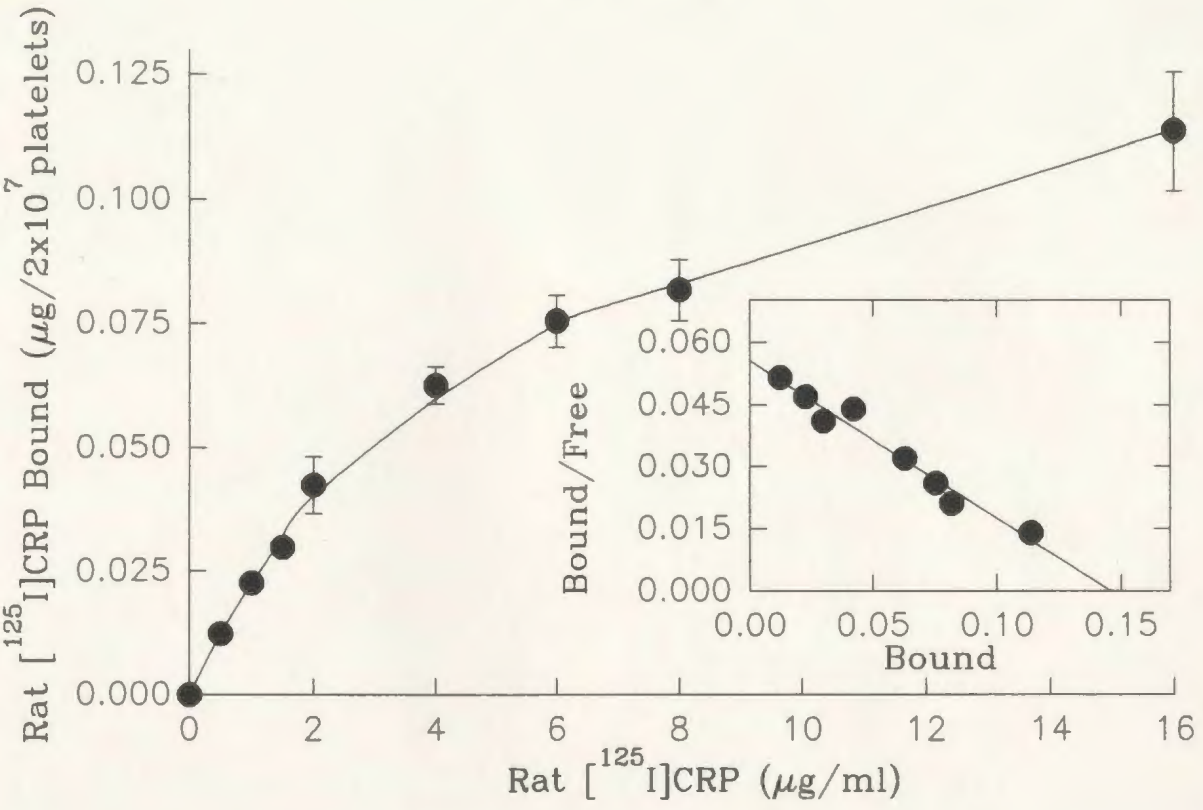
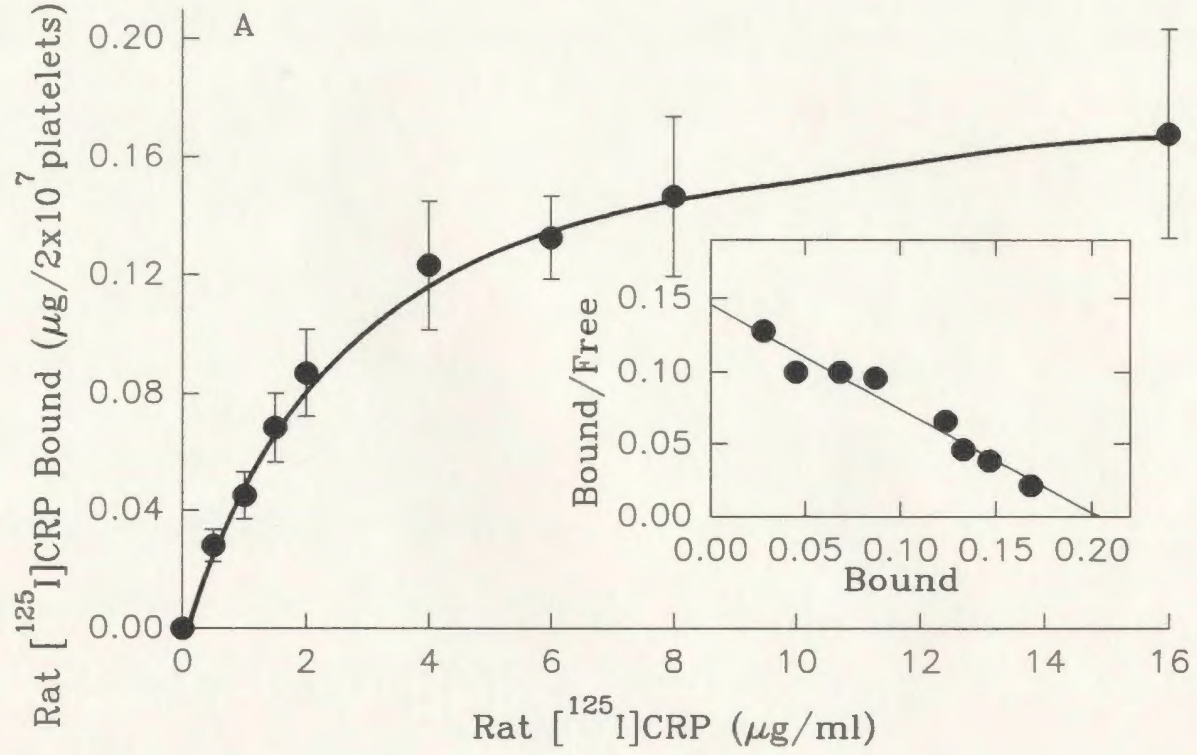
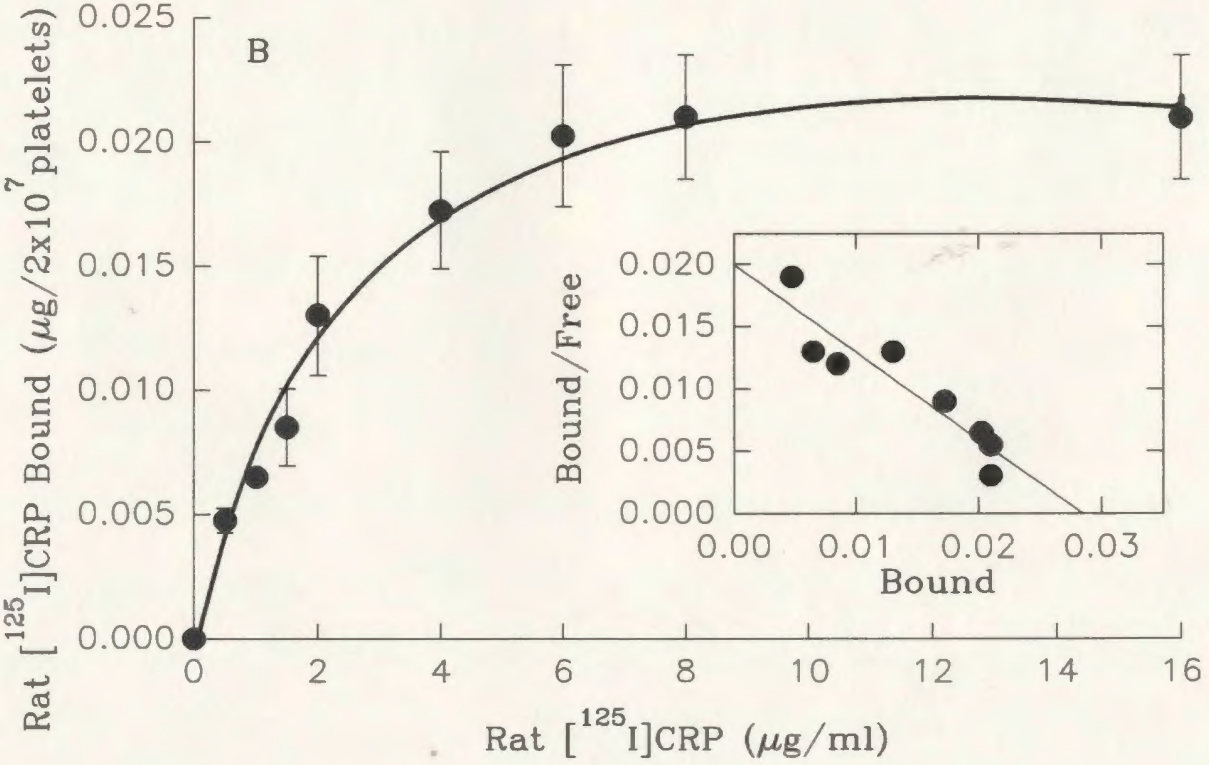


Fig. 3. **Specific binding of rat [^{125}I]CRP to rat platelets.** Binding studies were carried out with increasing concentrations of unlabelled rat CRP and fixed rat [^{125}I]CRP incubated for 30 min with washed rat platelets (4 x 10^7/ml) in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.25% BSA (w/v). Assay was stopped by centrifuging at 12,800 x g for 2 min. Platelet pellet was washed (1 x) in ice cold buffer and radioactivity associated with it was counted by γ-counter. Non-specific binding was determined by adding 25-400 fold excess of unlabelled rat CRP. Inset is scatchard plot analysis of the binding of rat [^{125}I]CRP to rat platelets. Data are mean $\pm$ S.D. for 4 separate experiments.

Fig. 4. **Saturable specific binding of rat [^{125}I]CRP to rabbit and human platelets.** Binding studies were carried out with increasing concentrations of unlabelled rat CRP and fixed rat [^{125}I]CRP incubated for 30 min with washed A) rabbit, or B) human platelets ($4x10^7$/ml) in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.25% BSA (w/v). Assay was stopped and the amount of rat [^{125}I]CRP bound to the platelets was determined as described to Fig. 3. Inset are scatchard plot analysis of the binding of rat [^{125}I]CRP to the platelets. Data are mean $\pm$ S.D. for 4 separate experiments.


A
Rat [125I]CRP Bound (μg/2x10^7 platelets)
0.20
0.16
0.12
0.08
0.04
0.00
0
2
4
6
8
10
12
14
16
Rat [125I]CRP (μg/ml)
Bound/Free
0.15
0.10
0.05
0.00
0.00 0.05 0.10 0.15 0.20
Bound


B
Rat [125I]CRP Bound (μg/2x10^7 platelets)
0.025
0.020
0.015
0.010
0.005
0.000
0
2
4
6
8
10
12
14
16
Rat [125I]CRP (μg/ml)
Bound/Free
0.020
0.015
0.010
0.005
0.000
0.00 0.01 0.02 0.03
Bound

rat [^{125}I]CRP binding sites on rat, rabbit and human platelets were similar (Table 1).

C. Inhibition of the Binding of Rat [^{125}I]CRP to Rat Platelets.

Phosphorylcholine, a ligand of rat CRP, inhibited and displaced the binding of rat CRP from rat platelets. Pre-incubation of increasing concentrations of phosphorylcholine (0-160 μM) with rat [^{125}I]CRP (2 μg/ml) for 10 min, followed by incubation with platelets for a further 30 min resulted in a dose-dependent inhibition of rat CRP-platelet binding with an IC_{50} value of 5.6 μM for phosphorylcholine (Fig. 5). Similarly, the addition of phosphorylcholine (0-50 μM) to binding assays in which platelets had been pre-incubated with rat [^{125}I]CRP (2 μg/ml) for a 30 min period resulted in the displacement of rat [^{125}I]CRP from rat platelets (Fig. 6).

The ability of unlabelled rat CRP to displace the binding of rat [^{125}I]CRP to rat platelets was also examined. These assays were carried out by adding increasing concentrations of unlabelled rat CRP (0-200 μg/ml) to platelets which had been pre-incubated with rat [^{125}I]CRP (2 μg/ml) for 30 min at 37 °C. In these studies a 100-fold molar excess of the unlabelled rat CRP almost completely displaced the binding of rat [^{125}I]CRP to platelets (Fig. 7).

The effect of unlabelled rabbit CRP on the binding of rat [^{125}I]CRP to rat platelets was also examined. These assays were carried out by incubating increasing concentrations of unlabelled rabbit CRP (0-100 μg/ml), rat [^{125}I]CRP (2 μg/ml) and platelets together at 37 °C for 30 min. Unlabelled rabbit CRP competed with rat

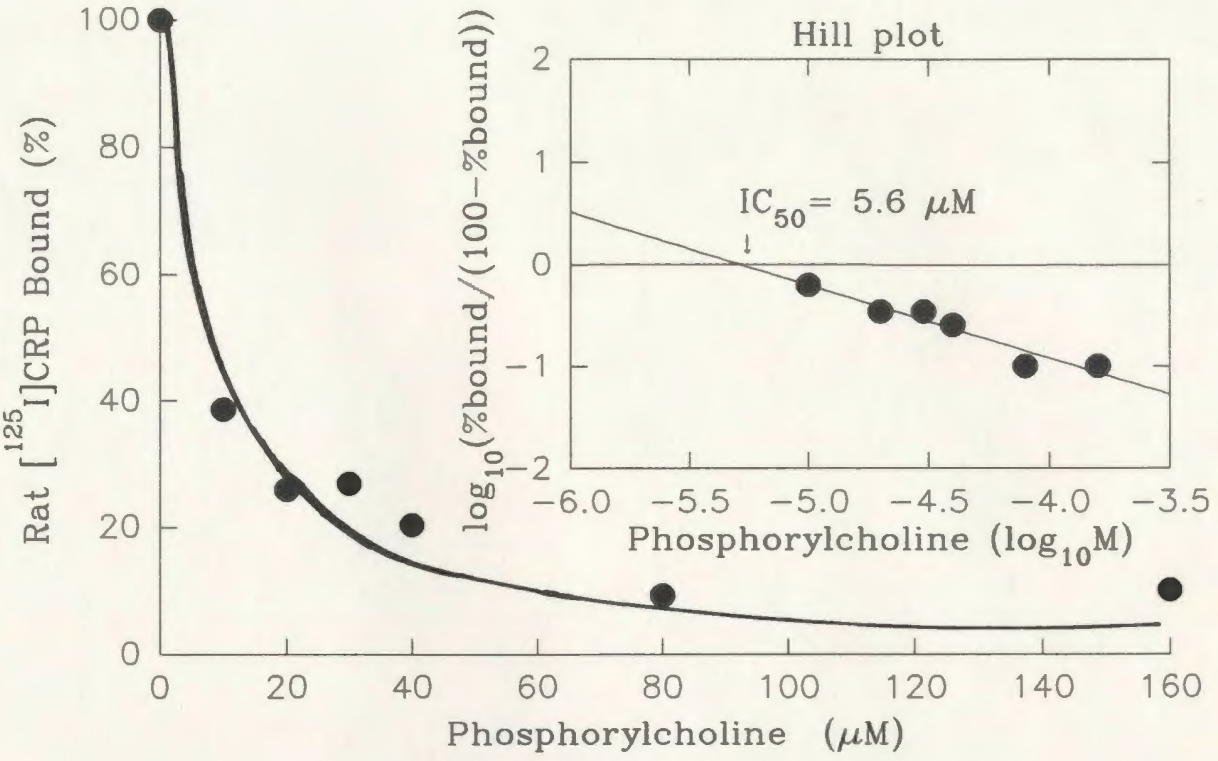


Fig. 5. **Effect of phosphorylcholine on binding of rat [^{125}I]CRP to rat platelets.** Rat [^{125}I]CRP (2 μg/ml) was incubated with different concentrations of phosphorylcholine (0-160 μM) for 10 min at room temperature. After this pre-incubation stage, platelets (4 x 10^7/ml) were added and incubated for a further 30 min at 37 °C. Assay was stopped and the amount of rat [^{125}I]CRP bound to the platelets was determined as described in Fig. 3. The amount of rat [^{125}I]CRP bound to the platelets in the absence of phosphorylcholine was defined as 100% bound. Data are mean of 2 experiments. Inset is a Hill plot of the binding data.

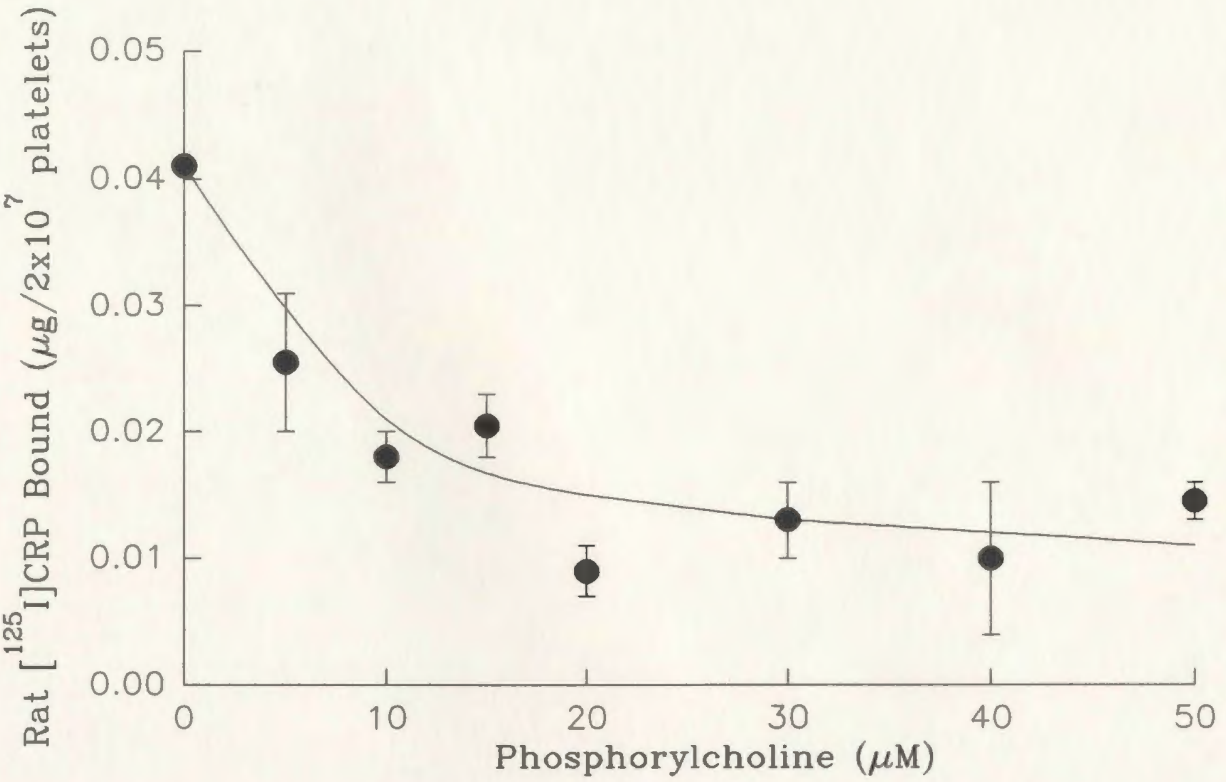


Fig. 6. **Displacement of the binding of rat [^{125}I]CRP to rat platelets by phosphorylcholine.** Rat [^{125}I]CRP (2 μg/ml) was incubated with platelets (4 x 10^7/ml) for 30 min. Phosphorylcholine (0-50 μM) was added and platelets were incubated for a further 10 min at 37 °C. Assay was stopped and the amount of rat [^{125}I]CRP bound to the platelets was determined as described for Fig. 3. Data are mean ± S.E. of 3 experiments.

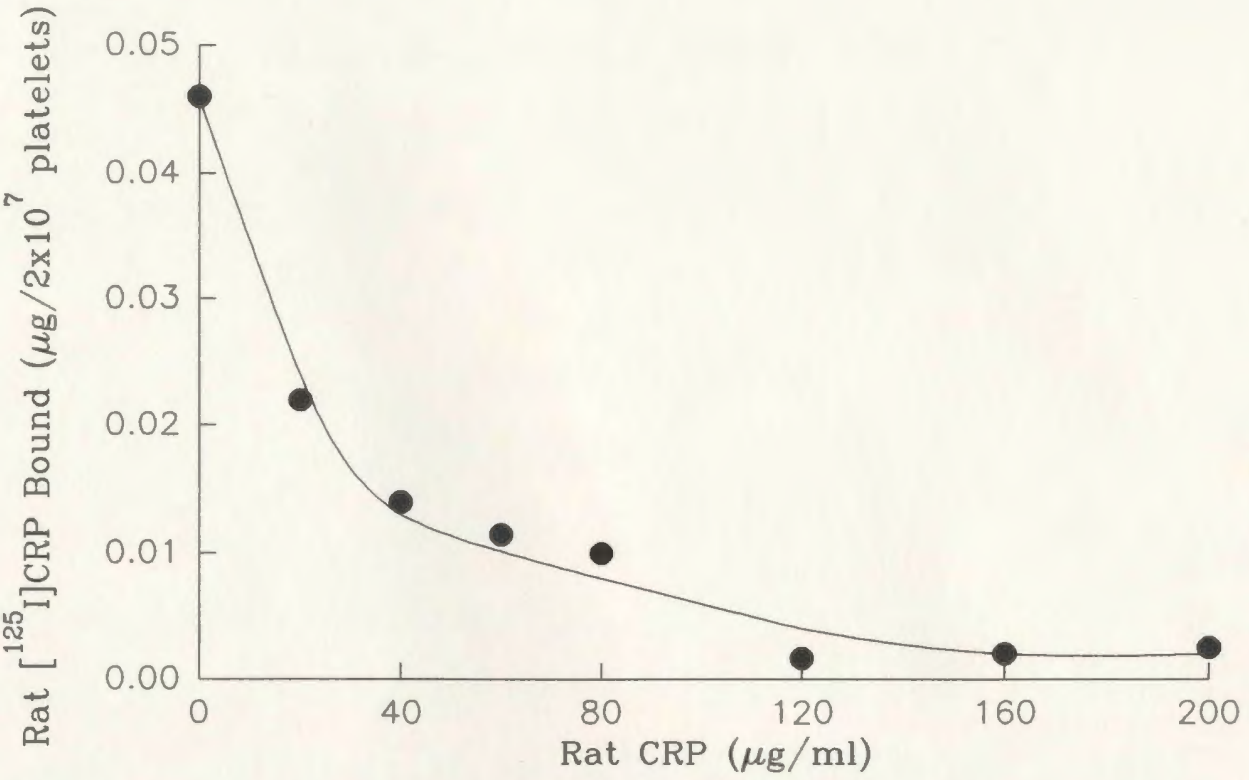


Fig. 7. **Displacement of the binding of rat [^{125}I]CRP to rat platelets by unlabelled rat CRP.** Rat [^{125}I]CRP (2 μg/ml) was incubated with platelets (4 x 10^7/ml) for 30 min. Unlabelled rat CRP (0-200 μg/ml) was added and platelets were incubated for a further 10 min at 37 ℃. Assays was stopped and the amount of rat [^{125}I]CRP bound to the platelets was determined as described for Fig. 3. Data are mean for 2 experiments.

[^{125}I]CRP for binding to platelets with an IC_{50} value of 1.6 μM for rabbit CRP (Fig. 8, inset). At the highest concentration used, rabbit CRP significantly inhibited ($p < 0.05$) the specific binding of rat [^{125}I]CRP to platelets by approximately 40%.

Section III. Discussion

In Chapter 5 of this thesis rat CRP was shown to inhibit *in vitro* aggregation of rat, rabbit and human platelets and it was apparent that during the inhibition of platelet aggregation a rat CRP-platelet interaction was involved. However, no previous study had characterized the binding of rat, rabbit or human CRP to platelets. Therefore, in order to prove a rat CRP-platelet interaction as a possible mechanism for inhibition of platelet aggregation, binding studies were carried out using rat [^{125}I]CRP and washed platelets from rat, rabbit and human plasma. The results of this binding study demonstrated a specific saturable binding of rat CRP to rat, rabbit and human platelets (Figs. 3 and 4). Scatchard analysis of the binding data revealed a single class of specific high affinity CRP binding sites on rat, rabbit and human platelets. The number of binding sites for rat CRP were greatest for rabbit platelets, followed by rat and human platelets (Table 1). This variation in the number of binding sites could be due to the differences in the actual number of binding sites present on the platelets or the availability of the binding sites for rat CRP. The concentration of rat CRP (16 μg/ml or 0.128 μM) which achieved near saturation of the binding sites on the platelets was within the range (0.04-0.48 μM) required for

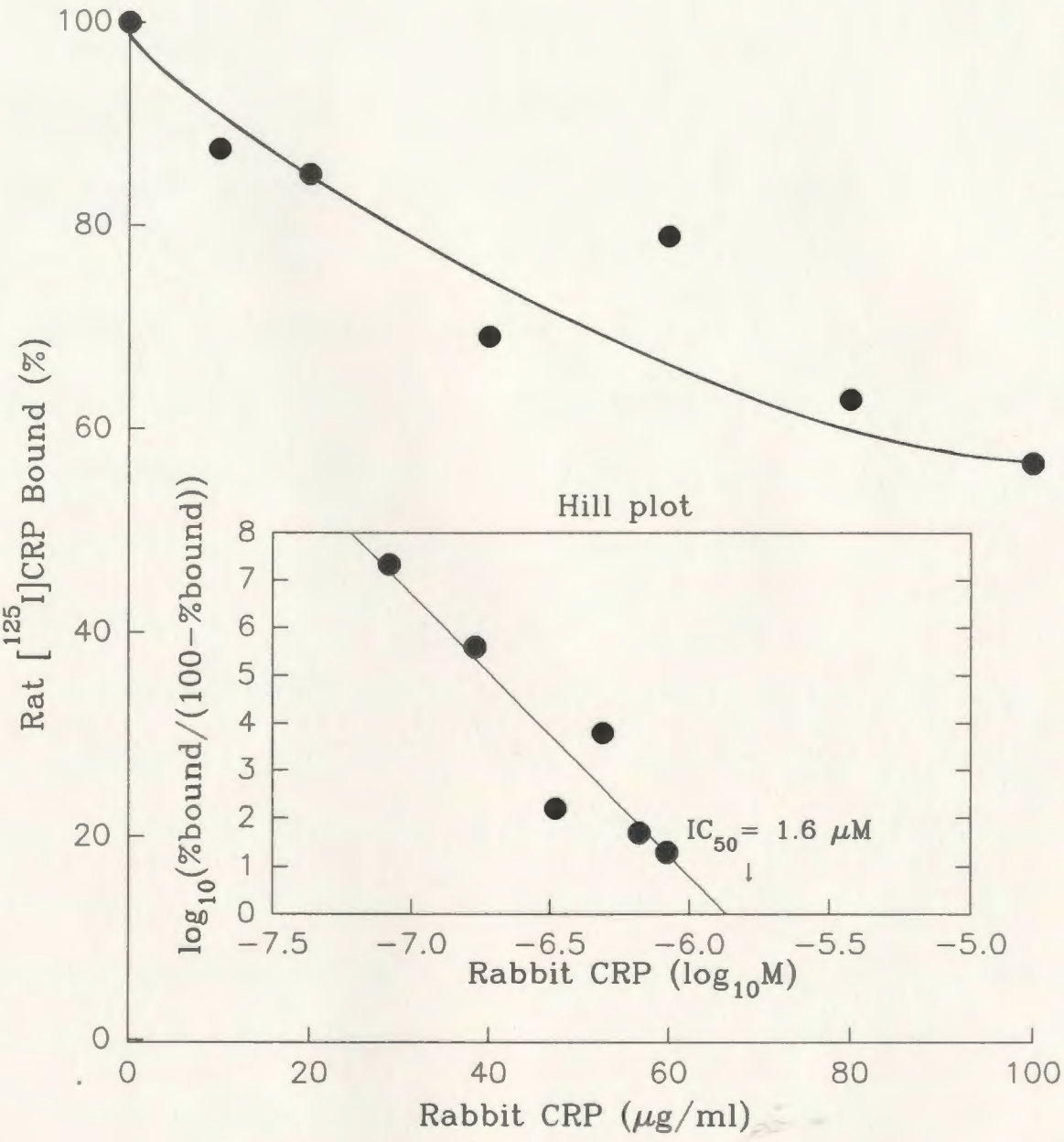


Fig. 8. **Effect of unlabelled rabbit CRP on the binding of rat [^{125}I]CRP to rat platelets.** Rat [^{125}I]CRP (2 μg/ml) and different concentrations of unlabelled rabbit CRP (0-100 μg/ml) were incubated with rat platelets (4 x 10^7/ml) for 30 min at 37 °C. Assays were stopped and the amount of rat [^{125}I]CRP bound to the platelets was determined as described for Fig. 3. The amount of rat [^{125}I]CRP bound to the platelets in the absence of rabbit CRP was defined as 100% bound. Data are mean for 2 experiments. Inset is a Hill plot of the binding data.

inhibition of platelet aggregation (Chapter 5).

The binding of rat CRP to platelets was time-dependent and required the presence of calcium in the incubation medium. Furthermore, this binding was inhibited by 50% in the presence of 5.6 μM phosphorylcholine (that is, IC_{50}= 5.6 μM). The inhibition of the binding of rat CRP to platelets in the presence of increasing concentrations of phosphorylcholine suggested the involvement of the phosphorylcholine-binding site on rat CRP in the binding process. This observation is consistent with the reversal of the inhibitory effect of rat CRP on ADP-induced platelet aggregation by phosphorylcholine.

The platelet-bound rat [^{125}I]CRP was displaced by the addition of either phosphorylcholine or unlabelled rat CRP. This displacement was dose-dependent and may suggest that the surface bound rat CRP was not modified as a consequence of the binding. Therefore, the inhibitory effect of rat CRP on platelet function appears to be mediated through reversible binding of rat CRP to sites on the outer surface of the plasma membrane.

The results suggest that the binding of rat CRP to platelets is specific, saturable and reversible. The binding of rat CRP to the surface of the platelet may be involved in the inhibition of platelet aggregation. Based on these observations the binding of rat CRP to platelets appeared to be receptor mediated. Furthermore, the results of experiments in which rabbit CRP competed with rat CRP for binding to platelets, suggest that the binding sites on platelets are common to both rat and rabbit

CRP. However, the observation that the inhibition was less than 50% in the presence of 100 μg/ml ($\sim$0.8 μM) rabbit CRP suggest that the binding affinity for rat CRP to rat platelets is greater than that for rabbit CRP.

In conclusion, the results of binding studies of rat CRP to platelets have shown that the binding of rat CRP to washed rat platelets may be receptor mediated. This is based on the observations that the rat CRP-platelet binding process met all the requirements for receptor mediated binding, viz., it is calcium-sensitive, saturable, specific and reversible by phosphorylcholine or unlabelled rat CRP and led to a physiological event which in this case was the inhibition of platelet aggregation. The binding of rat CRP to the platelet surface may play a role in the regulation of platelet response by the different biochemical pathways involved in signal transduction. This possibility was examined and is reported in the following chapter.

CHAPTER 7

STUDIES ON THE EFFECT OF RAT CRP ON PLATELET PHOSPHOLIPASES

Section I. Introduction

Agonist-induced stimulation of platelets result in platelet shape change, aggregation and secretion. These responses are often initiated by receptor-mediated activation of certain phospholipases, such as PI-PLC and PLA_2 (Huang and Detwiler, 1986). The role of PLA_2 in platelet function has been well documented in the literature (Huang and Detwiler, 1986; Lagarde, 1988; Needleman et al., 1986). In platelets, PI-PLC mediated hydrolysis of PIP_2 leads to generation of two signalling substances, IP_3 and DAG (Berridge, 1984; Berridge and Irvine, 1984). In the presence of calcium, DAG activates PKC which results in the phosphorylation of a number of proteins involved in the control of platelet cellular responses (Huang and Detwiler, 1986; Friesen and Gerrard, 1985).

Recent evidence suggesting agonist-induced DAG-generation from PC in a variety of tissues, has challenged the classical scheme involving inositol lipids as the only source of DAG during signal transduction (Billah and Anthes, 1990; Exton, 1990). DAG can be produced from PC by the action of PC-PLC or by a combined action of PLD and PA phosphohydrolase (Fig. 1) (Billah and Anthes, 1990; Exton,

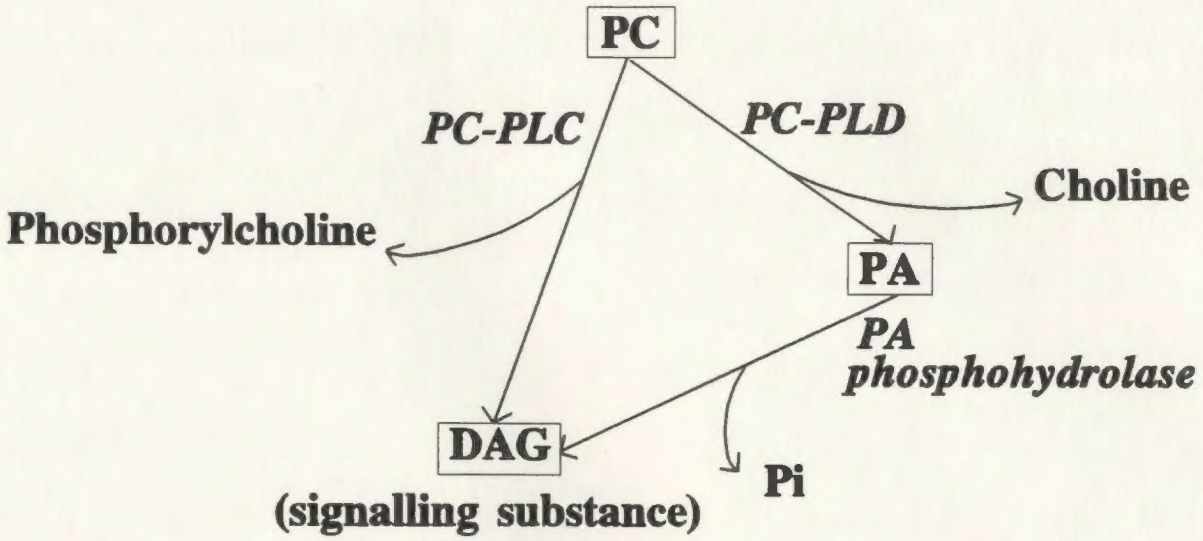


Fig. 1. **Mechanisms for formation of DAG by either PC-PLC or PLD/PA phosphohydrolase activity.**

1990). Studies (Rubin, 1988; Marche et al., 1990; Remmal et al., 1988; Nazih et al., 1990; Van Der Meulen and Haslam, 1990) have also reported the formation of radioactively labelled DAG and PA from labelled PC in activated platelets. However, none of these studies have conclusively shown the presence of PC-PLC enzyme in platelets. One purpose of the present study was to determine if PC-PLC activity was present in rat platelets.

The involvement of rat CRP in inhibiting ADP-, PAF-, and thrombin-induced platelet aggregation was discussed in Chapter 5. This inhibitory effect of rat CRP is believed to be mediated by the binding of rat CRP to its binding sites on platelets (Chapter 6). The purpose of this study was also to determine if any changes in the activity of platelet phospholipases, involved in signal transduction mechanisms, occurred as a consequence of the binding of rat CRP to the platelets.

Section II. Results

A. Evidence for PC-PLC activity in Rat Platelets

The presence of PC-PLC activity in rat platelet sonicates was examined using either [^{3}H-choline]DPPC or [2-^{3}H palmitoyl]DPPC vesicles as substrate. When rat platelet sonicates (from 5 x 10^7 cells/ml) were incubated with [^{3}H-choline]DPPC the formation of [^{3}H]phosphorylcholine resulted. Identification of this metabolite was made by TLC in methanol/0.9% NaCl/ammonium hydroxide (10/10/1, v/v/v), using authentic standards (Fig. 2A). The identification of [^{3}H]phosphorylcholine was also

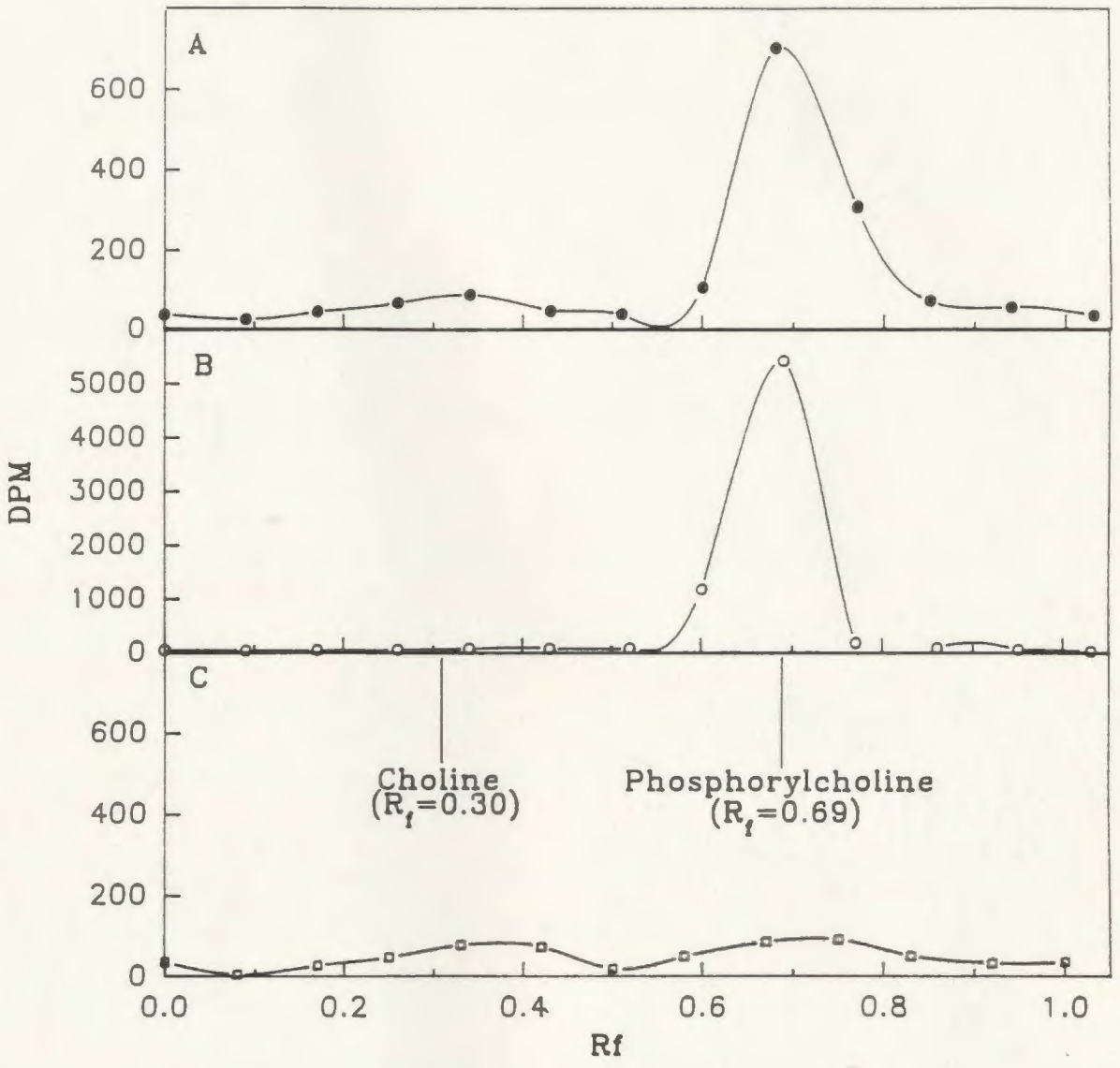


Fig. 2. **TLC of aqueous metabolites of [^{3}H-choline]DPPC.** [^{3}H-choline]DPPC (18 μM) was incubated for 40 min with A) rat platelet sonicates (5 x 10^7 cells/ml) or B) bacterial phospholipase C (0.02 U/ml). An aliquot (100 μl) of aqueous layer was applied to the plate and developed to separate the choline metabolites as described in Methods and Materials (section IV.C.1.). Each lane was divided into 1 cm segments which were scraped and counted. Panel C shows the profile in the absence of any enzyme. TLC profiles shown are typical of at least 5 profiles giving similar results.

confirmed by comparing the R_f of [^{3}H]phosphorylcholine formed by platelet sonicate PC-PLC with the R_f of [^{3}H]phosphorylcholine formed by the action of bacterial (*C. welchii*) phospholipase C on [^{3}H-choline]DPPC (Fig. 2B). Fig. 2C shows the TLC profile from control incubations of [^{3}H-choline]DPPC without any enzyme. Based on these results, the product of the [^{3}H-choline]DPPC hydrolysis by platelet sonicates was identified as [^{3}H]phosphorylcholine. Formation of [^{3}H]choline was not detected in these assays. When [2-^{3}H palmitoyl]DPPC and [^{3}H-choline]DPPC were used as substrates with platelet sonicates, the formation of [^{3}H]DAG and [^{3}H]phosphorylcholine were monitored as a function of time by TLC. The rate of formation of [^{3}H]DAG and [^{3}H]phosphorylcholine were similar (Fig. 3).

The production of [^{3}H]phosphorylcholine was dependent on the concentrations of [^{3}H-choline]DPPC and platelet sonicates (Fig. 4A and B). [^{3}H]Phosphorylcholine production increased with increasing concentrations of [^{3}H-choline]DPPC (Fig. 4A). Transformation of this data produced a linear double reciprocal plot (Fig. 4A, inset) which gave a Km of approximately 100 μM. Fig. 4B shows a linear increase of [^{3}H]phosphorylcholine formation with increasing platelet sonicate concentrations up to the equivalence of 10^8 platelets/ml.

Fig. 5 shows the platelet sonicate PC-PLC activity as a function of increasing calcium concentrations. The enzyme was active over the entire range of calcium concentrations (25 nM-25 mM) tested.

The PC-PLC activity in sonicated platelets from human or rabbit were also

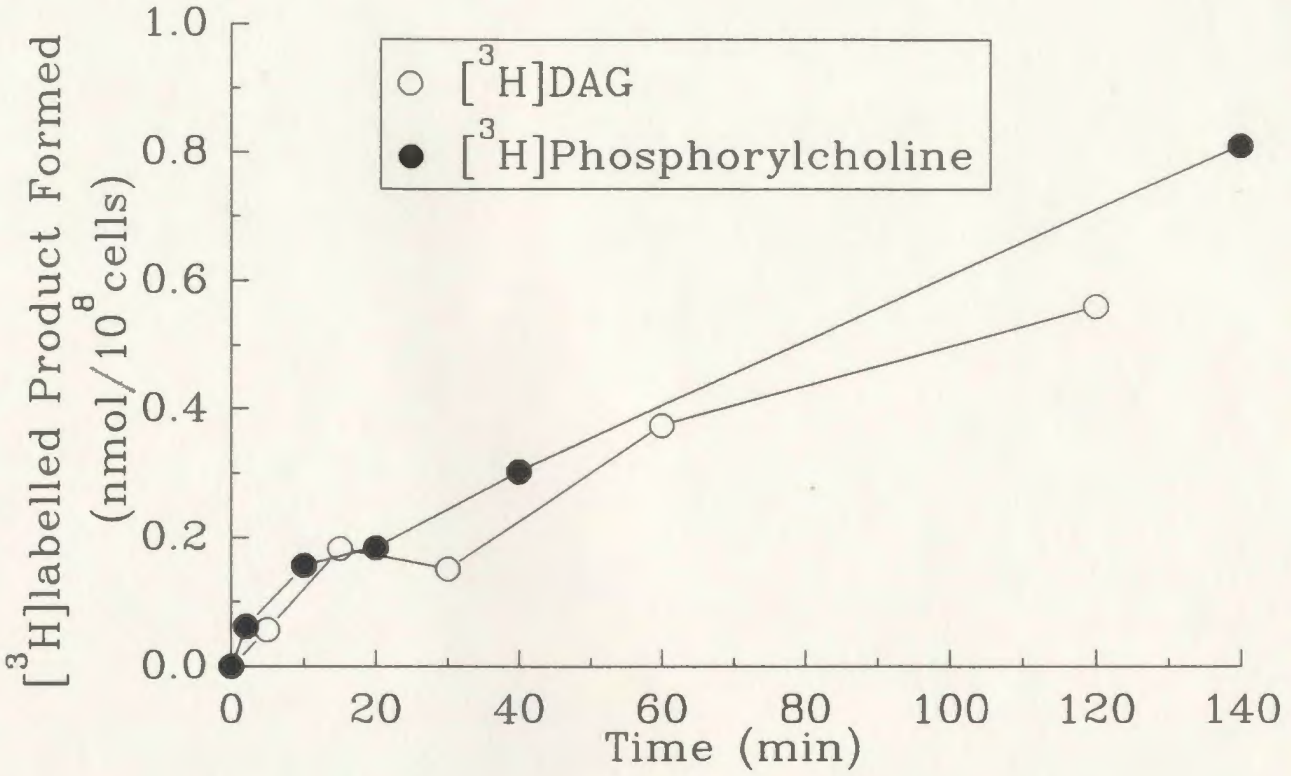
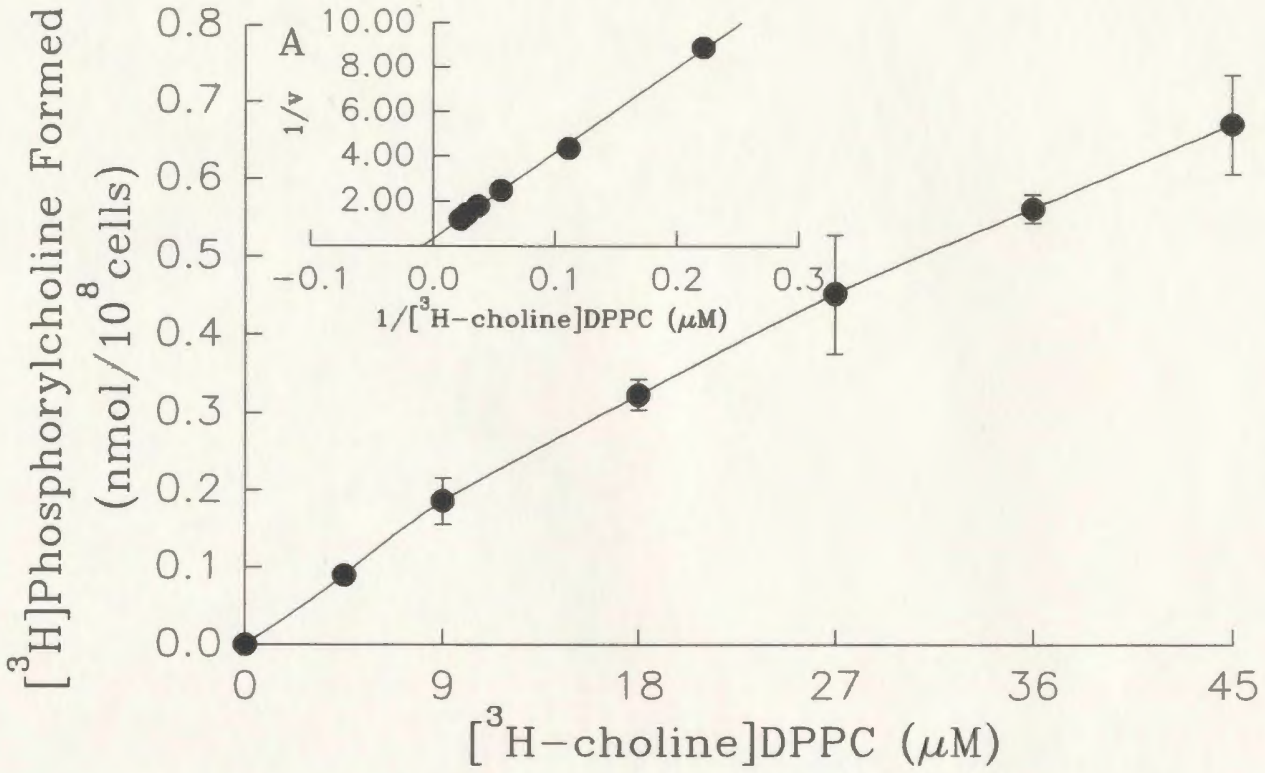
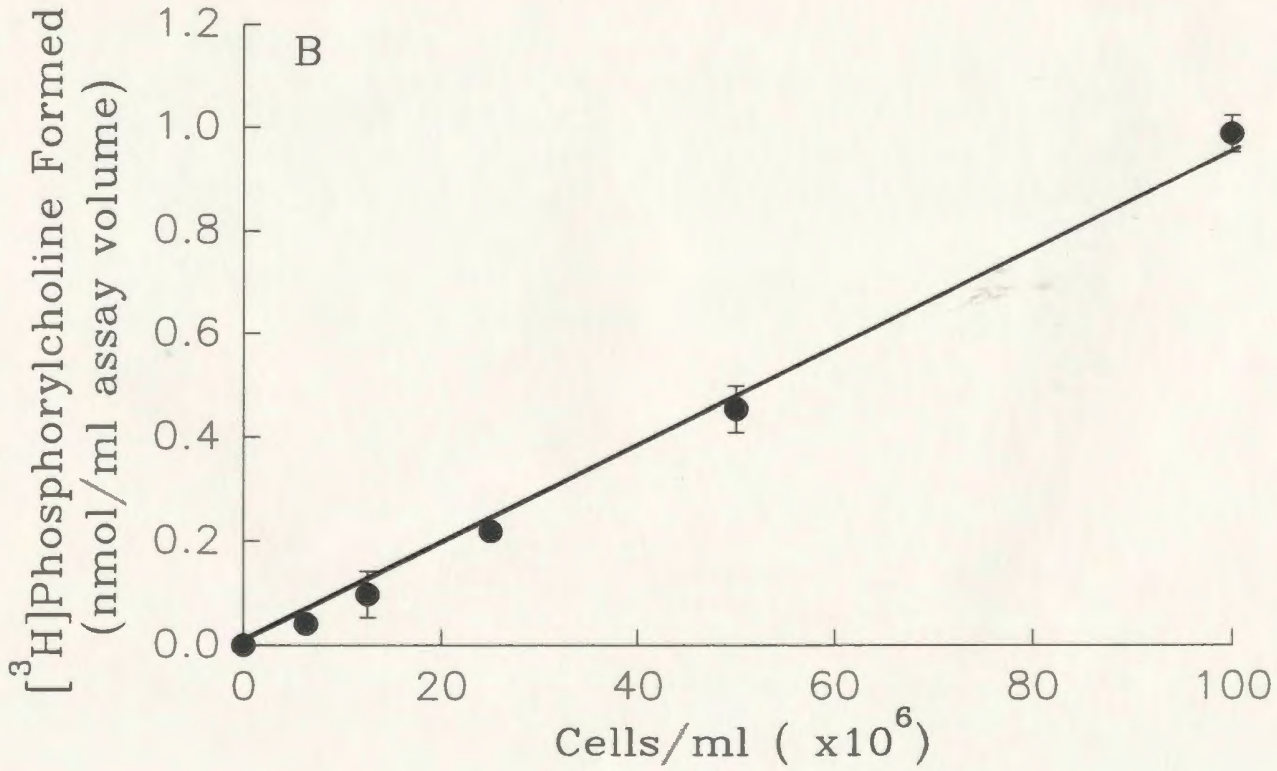


Fig. 3. **Time course of the formation of [^{3}H]DAG and [^{3}H]phosphorylcholine by rat platelet sonicates.** Platelet sonicates (5 x 10^7 cells/ml) were incubated with [2-^{3}H palmitoyl]DPPC (18 μM) or [^{3}H-choline]DPPC (18 μM). The reaction was stopped at the specified times and the aqueous and non-aqueous layers of extracted assays were analyzed by TLC as described in Methods and Materials (sections IV.C.1. and IV.C.4.). Data points are the mean of 2 determinations for [^{3}H]phosphorylcholine and 3 determinations for [^{3}H]DAG.

Fig. 4. **Formation of [^{3}H]phosphorylcholine as a function of [^{3}H-choline]DPPC and rat platelet sonicate concentration.** A) Platelet sonicates (5 x 10^7 cells/ml) were incubated with increasing concentrations of [^{3}H-choline]DPPC (0-45 μM). Inset is a double reciprocal plot of the substrate dose curve data. B) Increasing amounts of rat platelet sonicates (0-1 x 10^8 cells/ml) were incubated with 18 μM [^{3}H-choline]DPPC. Reactions were stopped after 40 min of incubation and the production of [^{3}H]phosphorylcholine was measured by TLC as described in Methods and Materials (section IV.C.1.). Data points are the mean $\pm$ S.E. of 3 determinations.


A
1/v
10.00
8.00
6.00
4.00
2.00
−0.1
0.0
0.1
0.2
0.3
1/[3H−choline]DPPC (μM)
[3H]Phosphorylcholine Formed
(nmol/10^8 cells)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
9
18
27
36
45
[3H−choline]DPPC (μM)


B
[3H]Phosphorylcholine Formed
(nmol/ml assay volume)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Cells/ml (x10^6)

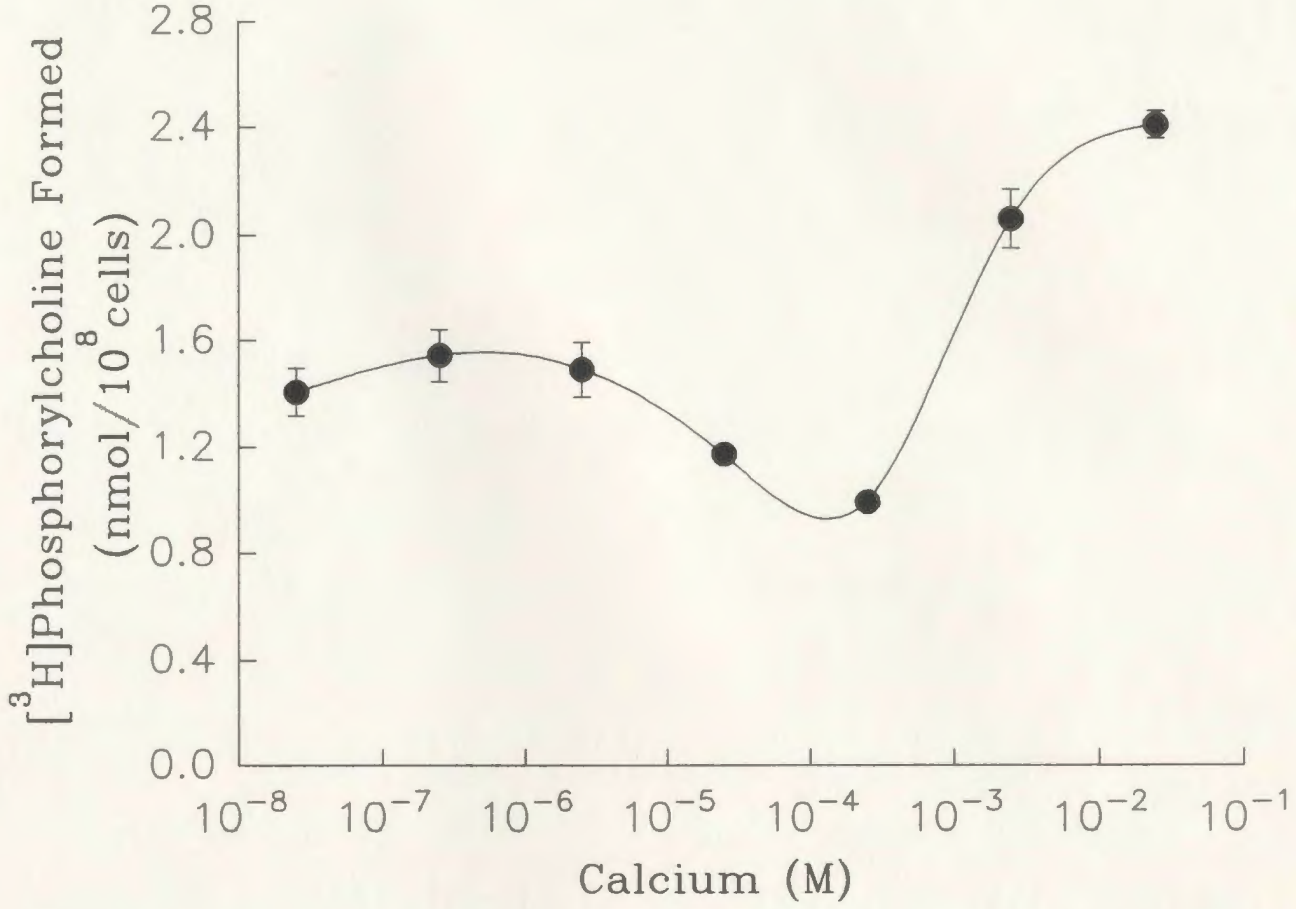


Fig. 5. **Effect of calcium concentration on formation of [^{3}H]phosphorylcholine from [^{3}H-choline]DPPC by rat platelet sonicates.** Platelet sonicates (5 x 10^7 cells/ml) were incubated with [^{3}H-choline]DPPC (18 μM) in presence of increasing concentrations of calcium (2.5 x 10^{-8}-2.5 x 10^{-2} M). Reactions were stopped after 40 min of incubation and the formation of [^{3}H]phosphorylcholine was measured by counting aqueous layers of extracted samples as described in Methods and Materials (section IX.C.). Data points are the mean $\pm$ S.E. of 3 determinations.

examined under optimized conditions. PC-PLC activity associated with the rat platelet sonicates was approximately 4-fold greater than the activity found in rabbit and human platelet sonicates (Fig. 6).

The effects of deoxycholate, Triton X-100, and oleate on PC-PLC activity in rat platelet sonicates were studied and the results are shown in Fig. 7. Deoxycholate (0.01%) increased PC-PLC activity by as much as 130% (2.3 times), while 0.01% Triton X-100 increased activity by 10 to 20% (1.1 to 1.2 times). Higher concentrations of deoxycholate, Triton X-100 or oleate inhibited the enzyme activity.

PC-PLC activity was assayed in platelet sonicates fractionated by ultracentrifugation at 105,000 x g for 1 h. The particulate and the supernatant fractions had almost equal PC-PLC activity (Fig. 8). The combined recovery of activities in the two fractions was greater than 75%. Both forms of the enzyme were inhibited by 2 mM EDTA when assayed in a calcium-free incubation medium (Fig. 9). The PC-PLC activity in the particulate fraction was increased by 0.01% deoxycholate and 0.01% Triton X-100, while, the PC-PLC activity in the supernatant was marginally increased by 0.01% deoxycholate but was inhibited by 0.01% Triton X-100.

Fig. 10 shows the pH profile of PC-PLC activity in platelet sonicates, particulate and supernatant fractions. The pH optima for PC-PLC activity in platelet sonicates, particulate and supernatant fraction were in the range of pH 7.2 to 7.6. The enzyme showed very low activity below pH 6.0.

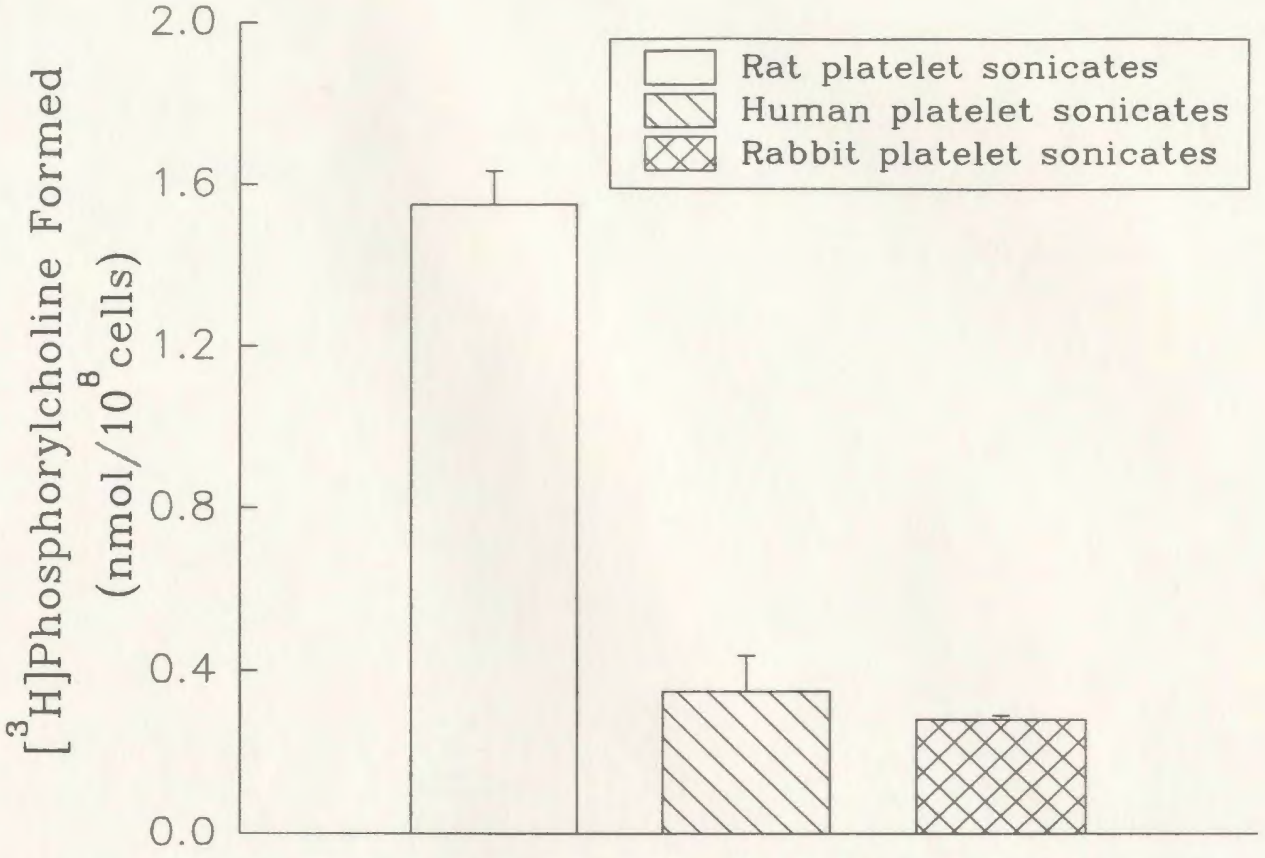
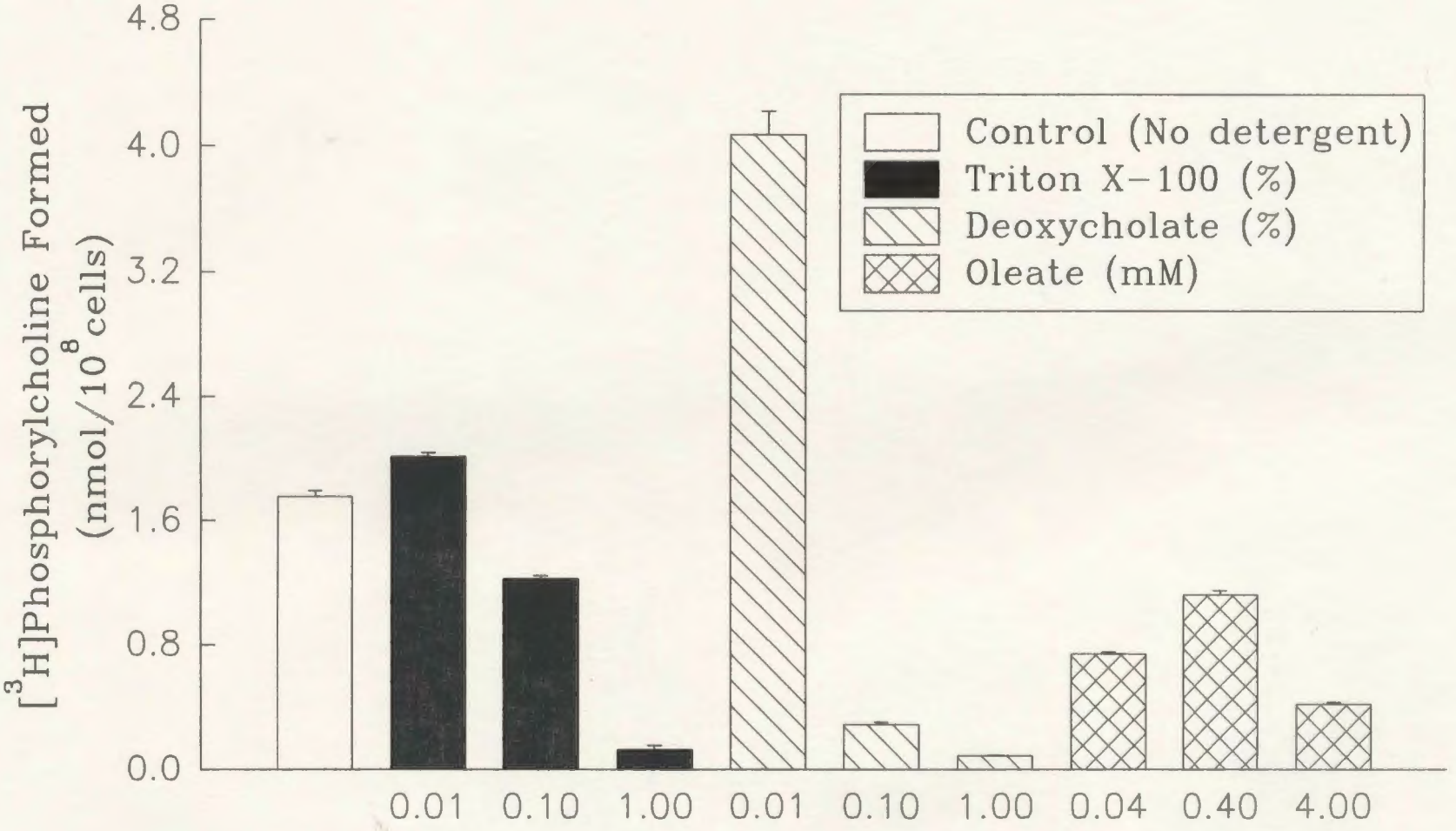


Fig. 6. **Comparison of PC-PLC activity of rat, rabbit and human platelet sonicates.** Platelet sonicates (5 x 10^7 cells/ml) from rat, rabbit, or human were incubated with [^{3}H-choline]DPPC (18 μM) for 20 min. Production of [^{3}H]phosphorylcholine was measured by TLC as described in Methods and Materials (section IV.C.1.). Values are the mean $\pm$ S.D. using 3 different individuals for each group.

Fig. 7. **Effect of detergents on [^{3}H]phosphorylcholine formation by rat platelet sonicates.** Platelet sonicates (5 x 10^7 cells/ml) were incubated with [^{3}H-choline]DPPC (18 μM) in presence or absence of deoxycholate, Triton X-100, or oleate. After 40 min the reactions were stopped, extracted, and analyzed by TLC for formation of [^{3}H]choline metabolites as described in Methods and Materials (section IV.C.1.). Values are the mean $\pm$ S.E. of 3 determinations.


4.8
4.0
3.2
2.4
1.6
0.8
0.0
[3H]Phosphorylcholine Formed
(nmol/10^8 cells)
Control (No detergent)
Triton X-100 (%)
Deoxycholate (%)
Oleate (mM)
0.01
0.10
1.00
0.01
0.10
1.00
0.04
0.40
4.00

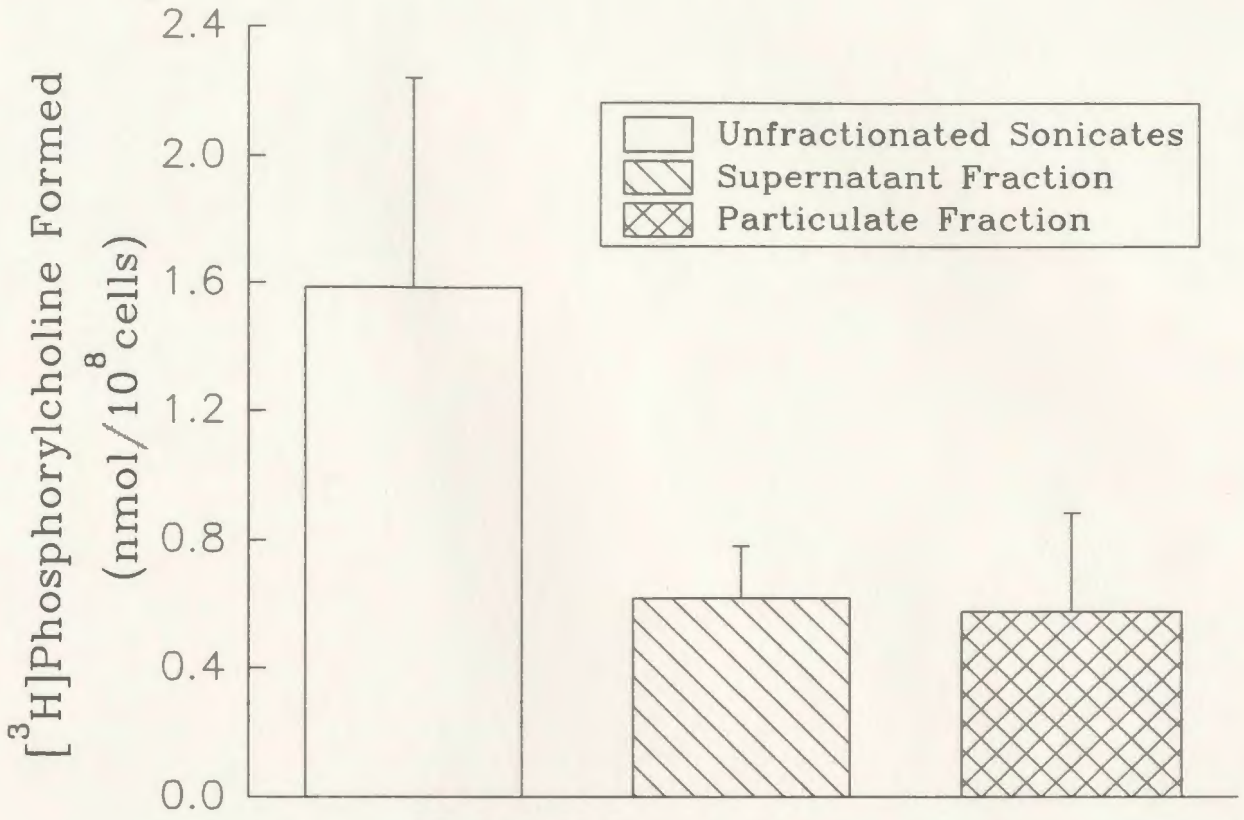


Fig. 8. **The distribution of PC-PLC activity in fractionated platelet sonicates.** Sonicated rat platelets (5 x 10^8/ml) were centrifuged (105,000 x g) in a Beckman Model L3-50 Ultracentrifuge for 60 min at 4 °C in Beckman Type 40 rotor. The supernatant fraction was removed and the pellet was re-suspended (particulate fraction), in a volume equal to the volume of the supernatant fraction, by agitation and sonication for three 5 sec intervals . Aliquots (40 μl) of the fractions were assayed by 40 min incubations using [^{3}H-choline]DPPC (18 μM) as described in Fig. 3. Values shown are mean $\pm$ S.E. of triplicate determinations using 2 animals.

Fig. 9. **Effect of detergents and EDTA on fractionated platelet sonicate PC-PLC activity.** Aliquots (40 μl) of the A) supernatant and B) particulate fractions of platelet sonicates prepared as described in Fig. 8 were assayed using [^{3}H-choline]DPPC (18 μM) in absence or presence of 0.01% deoxycholate, 0.01% Triton X-100, or 2 mM EDTA. After 40 min the reactions were stopped, extracted, and the aqueous layers were counted for radioactivity as described in Methods and Materials (section IX.C.). Values shown are mean $\pm$ S.E. of 3 determinations.

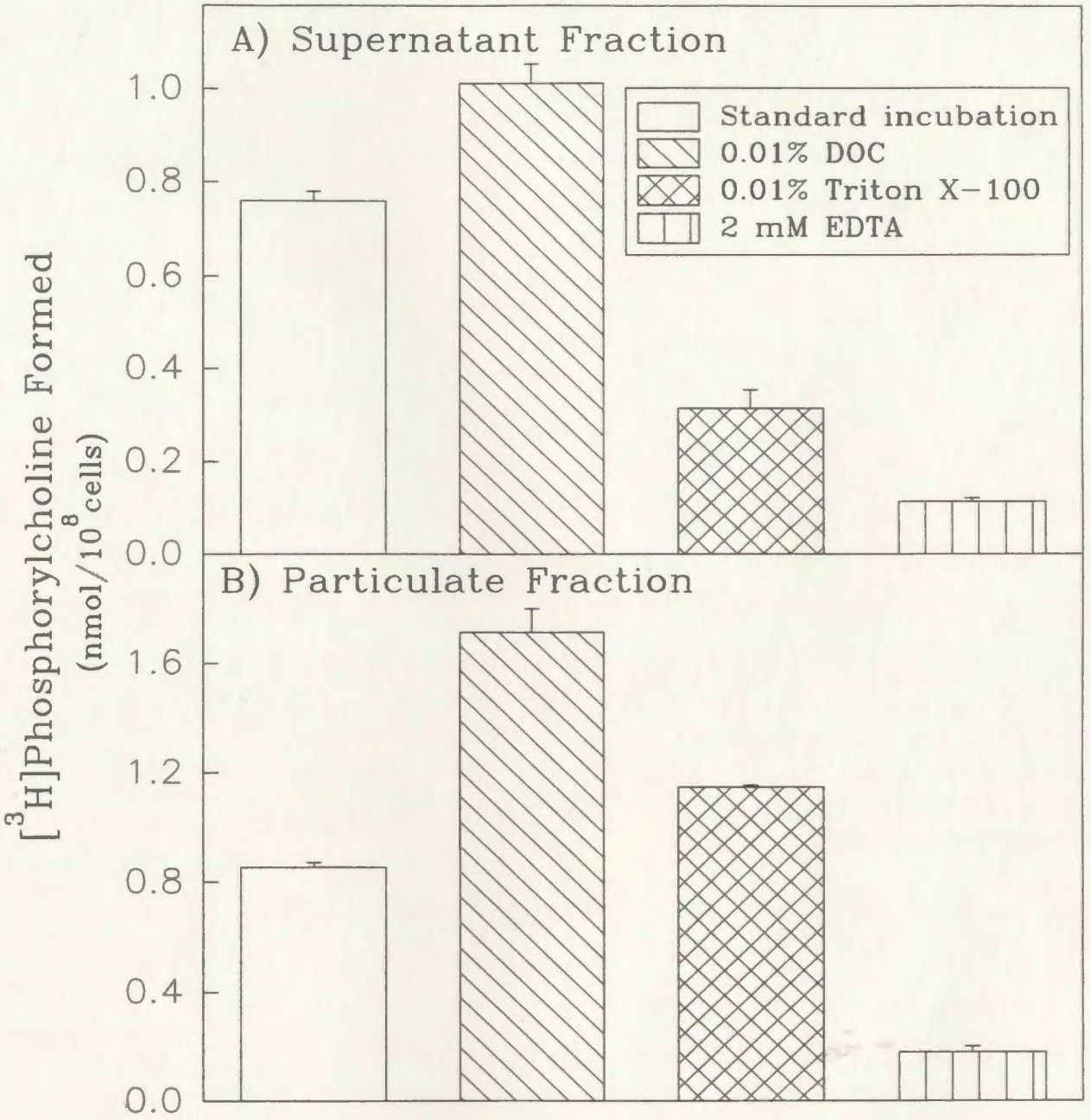

A) Supernatant Fraction
B) Particulate Fraction
Standard incubation
0.01% DOC
0.01% Triton X-100
2 mM EDTA
[3H]Phosphorylcholine Formed
(nmol/10^8 cells)
1.0
0.8
0.6
0.4
0.2
0.0
1.6
1.2
0.8
0.4
0.0

Fig. 10. **Effect of pH on formation of [^{3}H]phosphorylcholine from [^{3}H-choline]DPPC by whole and fractionated platelet sonicates.** A) Platelet sonicates (5 x 10^7 cells/ml) were incubated with increasing concentrations of [^{3}H-choline]DPPC (18 μM) for 40 min in buffered (pH 5.2-8.4) 50 mM Tris-maleate solution containing 2.5 mM $CaCl_2$. Aliquots (40 μl) of B) particulate and C) supernatant fraction were prepared and assayed as described in Fig. 8. Data points are the mean $\pm$ S.E. of 3 determinations.

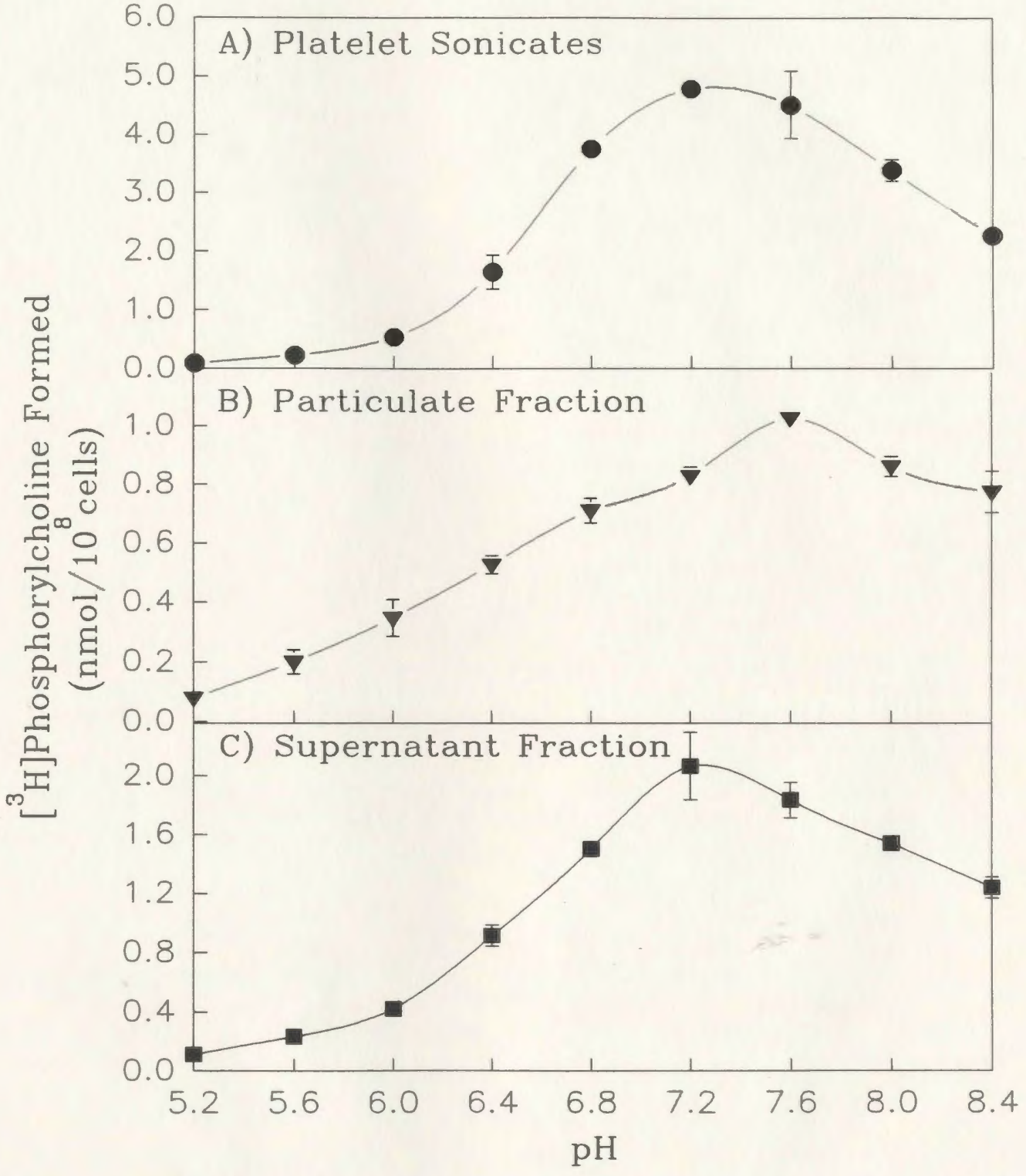

A) Platelet Sonicates
B) Particulate Fraction
C) Supernatant Fraction
[3H]Phosphorylcholine Formed (nmol/10^8 cells)
pH
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.8
0.6
0.4
0.2
1.6
1.2
5.2
5.6
6.0
6.4
6.8
7.2
7.6
8.0
8.4

Table 1. **Percentage incorporation of [^{3}H]myristic acid and [^{3}H]arachidonic acid into platelet lipids.** Statistics are mean ± S.D. for 4 separate experiments.

	Incorporation (%)	
lipids	[^{3}H]Myristic acid	[^{3}H]Arachidonic acid
PC	54.0±9.8	42.0±2.6
PE	8.8±2.7	38.1±2.2
PS & PI	2.3±0.4	16.0±3.0
PA	0.4±0.1	1.7±0.4
DAG	1.8±0.6	0.3±0.1
Others*	32.3±7.8	1.9±0.4

*Composed of monoacylglyerol, triacylglycerol, free fatty acid, alkyldiacylglyerol and cholesterol ester.

In order to determine if the activity of platelet PC-PLC could be affected by thrombin, platelets labelled with [^{3}H]myristic acid or [N-*methyl*-^{14}C]lyso-PAF were used. Labelling platelets with radioactive lyso-PAF has been previously shown to enrich the platelet PC pool (Huang et al., 1991). Table 1 compares the incorporation of the [^{3}H]-label into lipids when platelets were labelled with either [^{3}H]myristic acid or [^{3}H]arachidonic acid. The enrichment of [^{3}H]myristic acid in the PC pool agrees well with the previously published results of Huang and Cabot (1990) who labelled

neutrophils with [^{3}H]myristic acid.

When [^{3}H]myristic acid-labelled platelets were stimulated by thrombin, there was a time-dependent formation of [^{3}H]DAG, which plateaued after approximately 120 sec (Fig. 11). The amount of [^{3}H]DAG formed when platelets were stimulated by 2 U/ml thrombin was about 1 % of the total labelled lipids. Since only 50% of the [^{3}H]myristic acid is incorporated in to PC (Table 1), it is possible that as much as 2% of total labelled PC was hydrolysed by platelet PC-PLC. A similar result has been reported with fibroblasts stimulated by bradykinin, in which about 2% of PC was hydrolysed to form DAG (Van Blitterswijk et al., 1991).

In order to confirm that the formation of DAG was from PC hydrolysis, [N-*methyl*-^{14}C]lyso-PAF-labelled platelets were used. The action of PC-PLC in [N-*methyl*-^{14}C]lyso-PAF-labelled platelets would result in the formation of [^{14}C]phosphorylcholine. A time-dependent formation of [^{14}C]phosphorylcholine from [N-*methyl*-^{14}C]lyso-PAF-labelled platelets with the addition of thrombin (2 U/ml) is shown in Fig. 12. The kinetics of the formation of [^{14}C]phosphorylcholine was similar to that of [^{3}H]DAG formation from platelets labelled by [^{3}H]myristic acid (Fig. 11). The formation of [^{14}C]phosphorylcholine as a function of increasing concentrations of thrombin (0-2 U/ml) is shown in Fig. 13. In some experiments, the treatment of platelets with 0.05 U/ml thrombin resulted in a net utilization of water-soluble [^{14}C]choline metabolites below basal levels. This may have been the result of increased synthesis of PC and utilization of background [^{14}C]phosphorylcholine during

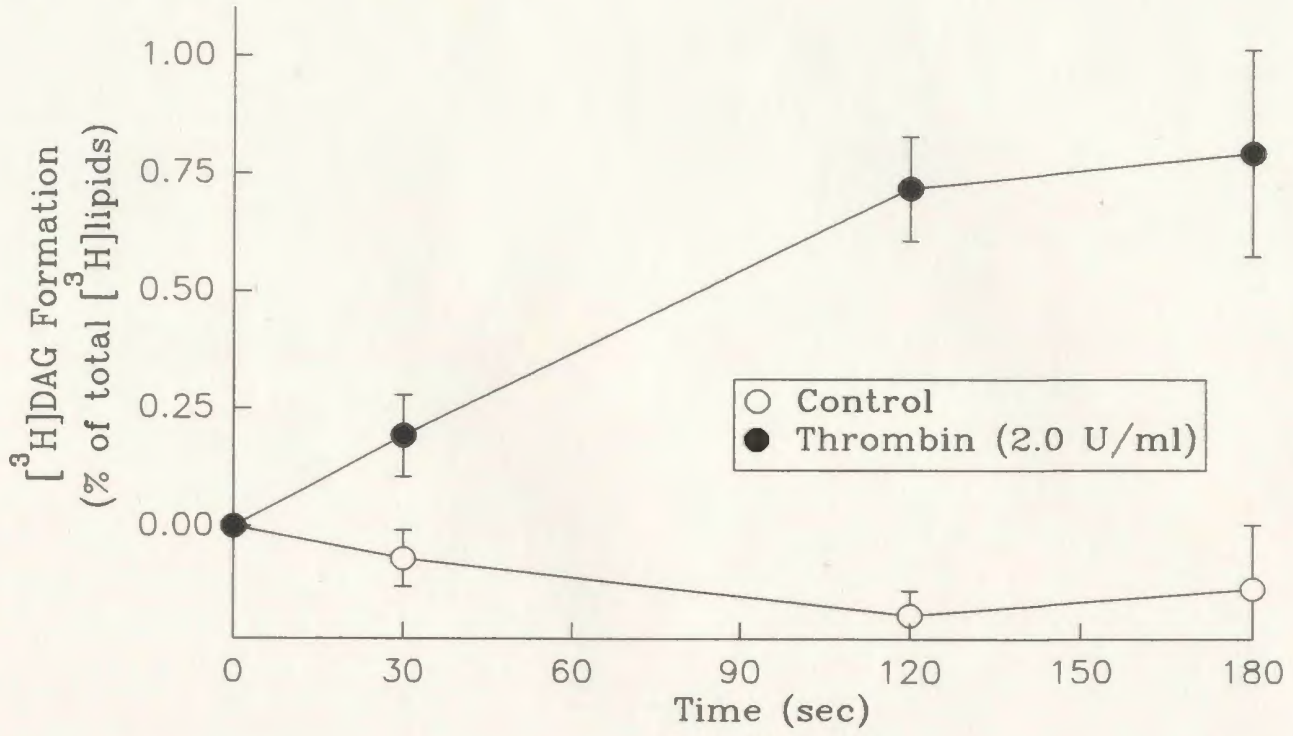


Fig. 11. **Time-dependence of the formation of [^{3}H]DAG from thrombin-stimulated platelets labelled with [^{3}H]myristic acid.** Platelets (5 x 10^8/ml), labelled with [^{3}H]myristic acid, were incubated in the absence (○) or presence of thrombin (2 U/ml-●) for up to 180 sec. At the indicated times the reaction was stopped by chloroform/methanol and the organic phase containing [^{3}H]DAG was analyzed by TLC as described in Methods and Materials (section IV.C.4). Each point is the mean ± S.E. of 4 separate experiments. The formation of [^{3}H]DAG is expressed as percent of total labelled lipid. The background value for [^{3}H]DAG present before the addition of thrombin was subtracted from the individual measurements.

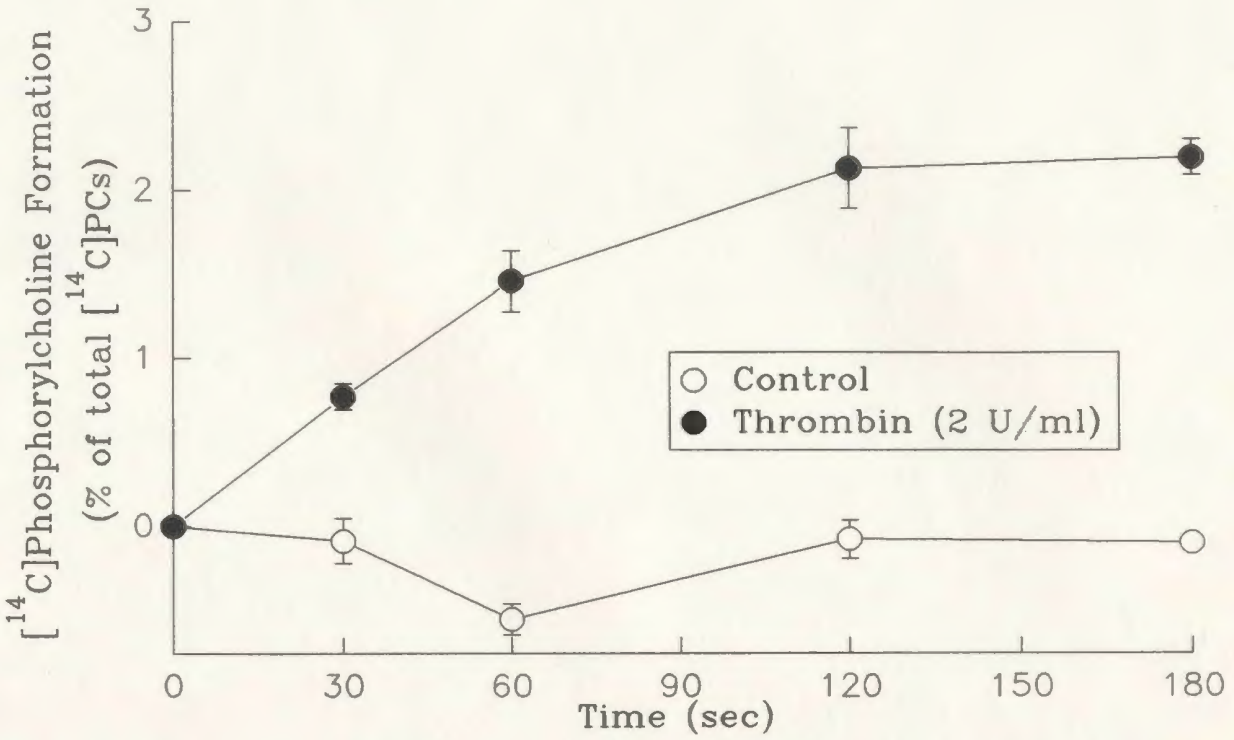


Fig. 12. **Time-dependence of the formation of [^{14}C]phosphorylcholine from thrombin-stimulated platelets labelled with [N-*methyl*-^{14}C]lyso-PAF.** Platelets (5 x 10^8/ml), labelled with [N-*methyl*-^{14}C]lyso-PAF, were incubated in the absence (○) or presence (●) of thrombin (2.0 U/ml) for up to 180 sec. The reactions were stopped by chloroform/methanol and extracted (Bligh and Dyer, 1959). Aqueous extracts containing [^{14}C]phosphorylcholine were counted for radioactivity. The formation of [^{14}C]phosphorylcholine is expressed as percent of total labelled lipid. The background value for [^{14}C]phosphorylcholine present before the addition of thrombin was subtracted from the individual measurements. Values are mean ± S.E. from 4 separate experiments.

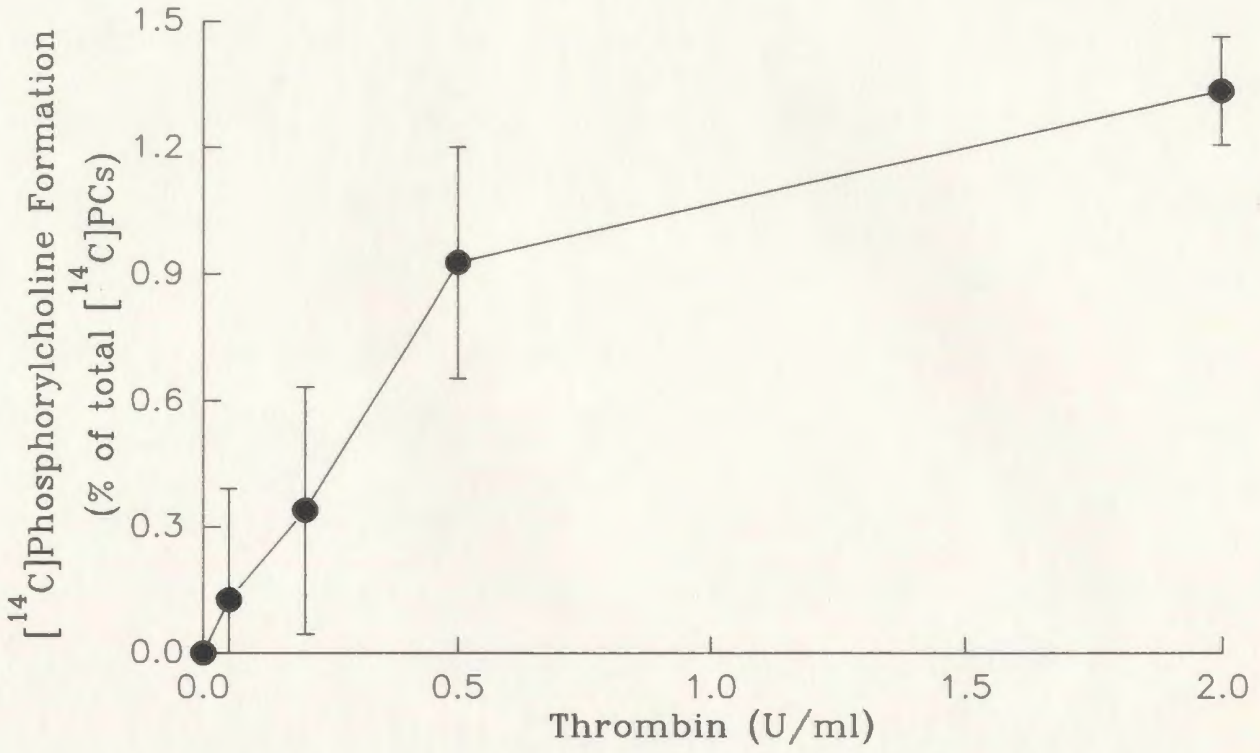


Fig. 13. **Effect of thrombin concentration on [^{14}C]phosphorylcholine formation from platelets labelled with [N-*methyl*-^{14}C]lyso-PAF.** Washed rat platelets, pre-labelled with [N-*methyl*-^{14}C]lyso-PAF, were exposed to thrombin (0.0-2.0 U/ml) for exactly 3 min. Incubations without added thrombin were also carried out and the results were subtracted, as blanks, from the results of assays with thrombin. The formation of [^{14}C]phosphorylcholine was measured as described in Fig. 12. Values are mean $\pm$ S.E. for 4 experiments.

stimulation (Remmal et al., 1988). However, higher doses of thrombin resulted in a net formation of [^{14}C]phosphorylcholine in platelets. There was no formation of either [^{14}C]phosphorylcholine or [^{3}H]DAG from platelets in the absence of thrombin. These results strongly suggests that DAG and phosphorylcholine are formed by the action of PC-PLC on PC in thrombin-stimulated rat platelets.

Although [N-*methyl*-^{14}C]lyso-PAF-labelled platelets were exhausively washed, whole lipid extraction of the unactivated platelets revealed approximately 20-25% of the total radioactivity from the platelets to be free [^{14}C]phosphorylcholine. The formation of a substantial labelled pool of phosphorylcholine in platelets during labelling procedures using [^{3}H]choline have been reported by others (Nakashima et al., 1991). Because of the high background levels of [^{14}C]phosphorylcholine, expression of PC hydrolysis in terms of % increase above control levels would not provide an accurate picture of the actual stimulation of PC hydrolysis by PC-PLC and the corresponding production of DAG. Therefore, expression of [^{14}C]phosphorylcholine formation in terms of % hydrolysis of [^{14}C]PC (total), after subtracting background, was used to estimate of the stimulation of PC-PLC by thrombin.

B. Effect on Rat CRP on Thrombin-Induced Platelet Aggregation and [^{14}C]Serotonin Secretion.

The effect of rat CRP on thrombin-induced platelet aggregation and serotonin

secretion was studied using [^{14}C]serotonin-loaded platelets. The inhibitory effect of rat CRP on [^{14}C]serotonin secretion, induced by 0.1 U/ml thrombin, was dependent on the concentration of rat CRP used (Fig. 14). The maximal inhibitory effect of rat CRP on thrombin-induced secretion of [^{14}C]serotonin was observed at 0.48 μM rat CRP. Higher concentrations of rat CRP (up to 1.6 μM) did not further increase the inhibitory effect.

The effect of rat CRP on platelet aggregation and [^{14}C]serotonin secretion as a function of thrombin concentration was examined (Figs. 15 and 16). Platelet aggregation was dependent on the concentration of thrombin used to stimulate the platelets. Maximum aggregation occurred with 0.5-2 U/ml thrombin (Fig. 15). Rat CRP (0.48 μM) had no effect on platelet aggregation when 0.2-2 U/ml thrombin was used. In contrast, platelet aggregation was inhibited by rat CRP when either 0.1 or 0.05 U/ml of thrombin was used to stimulate the platelets (Fig. 15). Similarly, the release of [^{14}C]serotonin from platelets was also dependent on the thrombin concentration (Fig. 16). Approximately 90% of the platelet-incorporated [^{14}C]serotonin was secreted in the presence of 2 U/ml thrombin which represented maximum secretory response. The percentage [^{14}C]serotonin secretion in the presence of 0.1 and 0.05 U/ml thrombin was approximately 35% and 10%, respectively (Fig. 16). Rat CRP significantly inhibited ($p<0.05$) the secretion of [^{14}C]serotonin by approximately 35% and 60% when 0.1 and 0.05 U/ml of thrombin was used, respectively.

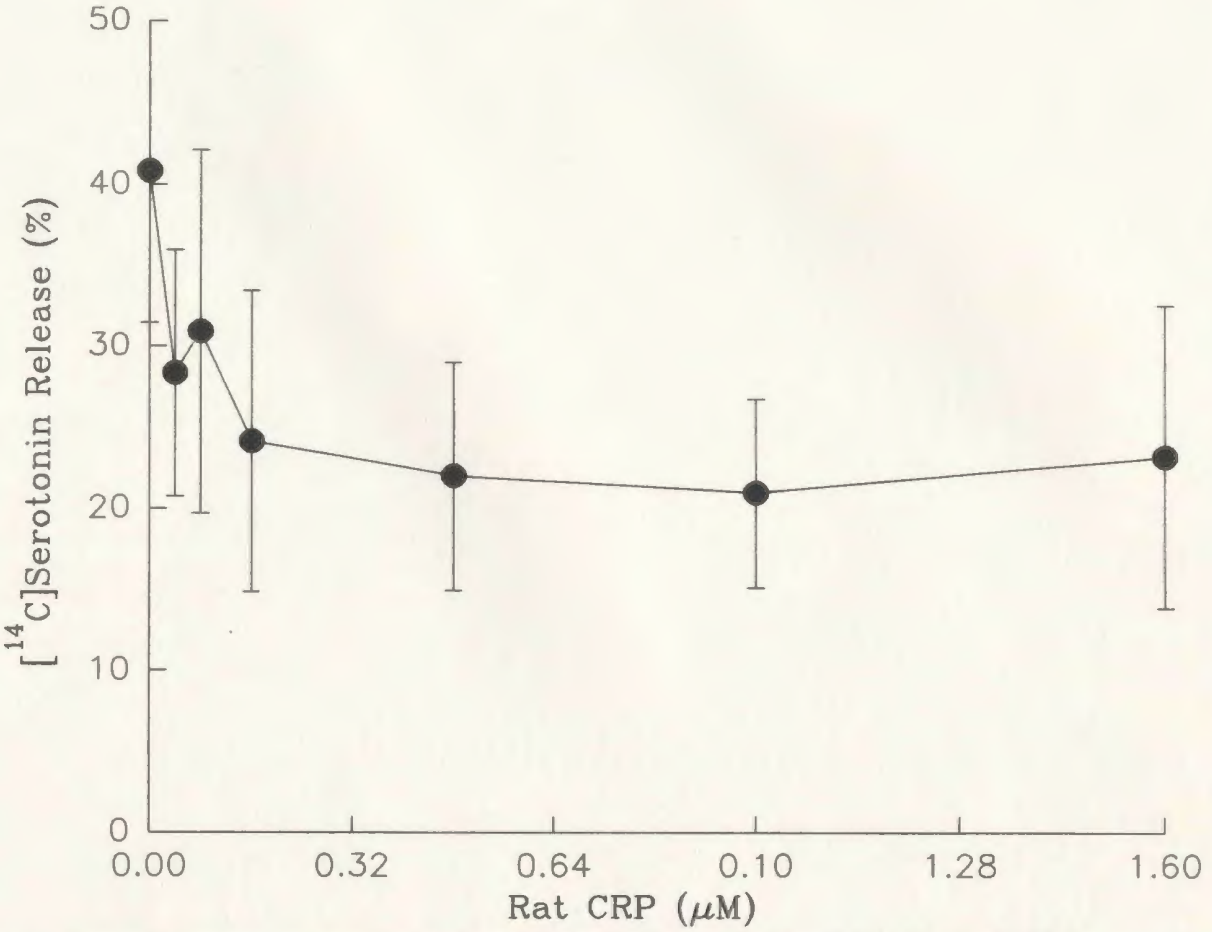
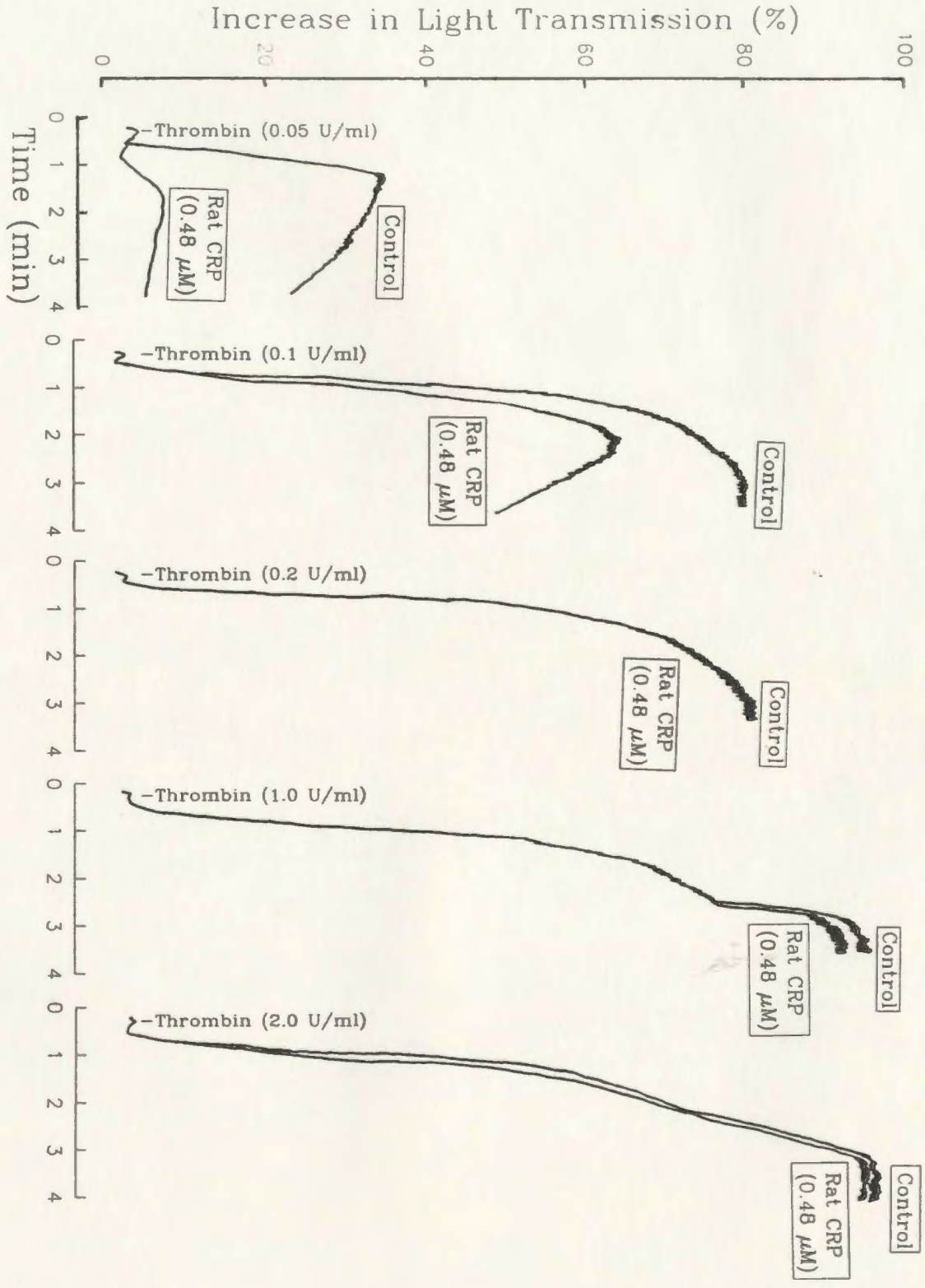


Fig. 14. **The influence of increasing concentrations of rat CRP on thrombin-induced platelet [^{14}C]serotonin secretion.** Washed platelets (4 x 10^8 platelets/ml) pre-labelled with [^{14}C]serotonin were incubated in presence of increasing concentrations of rat CRP (0-1.6 μM) for 15 min, prior to stimulation with thrombin (0.1 U/ml). Platelets were allowed to aggregate for exactly 3.0 min and then 100 μl of mixture was transferred to an eppendorf tube containing 25 μl of ice-cold formaldehyde (0.63 M)-EDTA (50 mM) solution to stop secretion. The mixture was centrifuged and the supernatant counted for radioactivity as described in Methods and Materials (section V.B.2.). Results are expressed as percentage of the maximum serotonin release. Results are mean $\pm$ S.E. for 4 experiments.

Fig. 15. **Effect of rat CRP on thrombin-induced platelet aggregation.** Washed rat platelets (4 x 10^8/ml) suspended in Tyrode solution (pH 7.4) were incubated in absence or presence of rat CRP (0.48 μM) at 37 °C. Platelet aggregation was measured for 3 min after increasing concentrations of thrombin (0.05-2 U/ml) were added to the suspension with constant stirring. 100% platelet aggregation was defined as maximum aggregation induced by 2 U/ml thrombin. Results are typical of 4 experiments.


Increase in Light Transmission (%)
0
20
40
60
80
100
Time (min)
0 1 2 3 4
-Thrombin (0.05 U/ml)
Rat CRP (0.48 μM)
Control
-Thrombin (0.1 U/ml)
Rat CRP (0.48 μM)
Control
-Thrombin (0.2 U/ml)
Rat CRP (0.48 μM)
Control
-Thrombin (1.0 U/ml)
Rat CRP (0.48 μM)
Control
-Thrombin (2.0 U/ml)
Rat CRP (0.48 μM)
Control

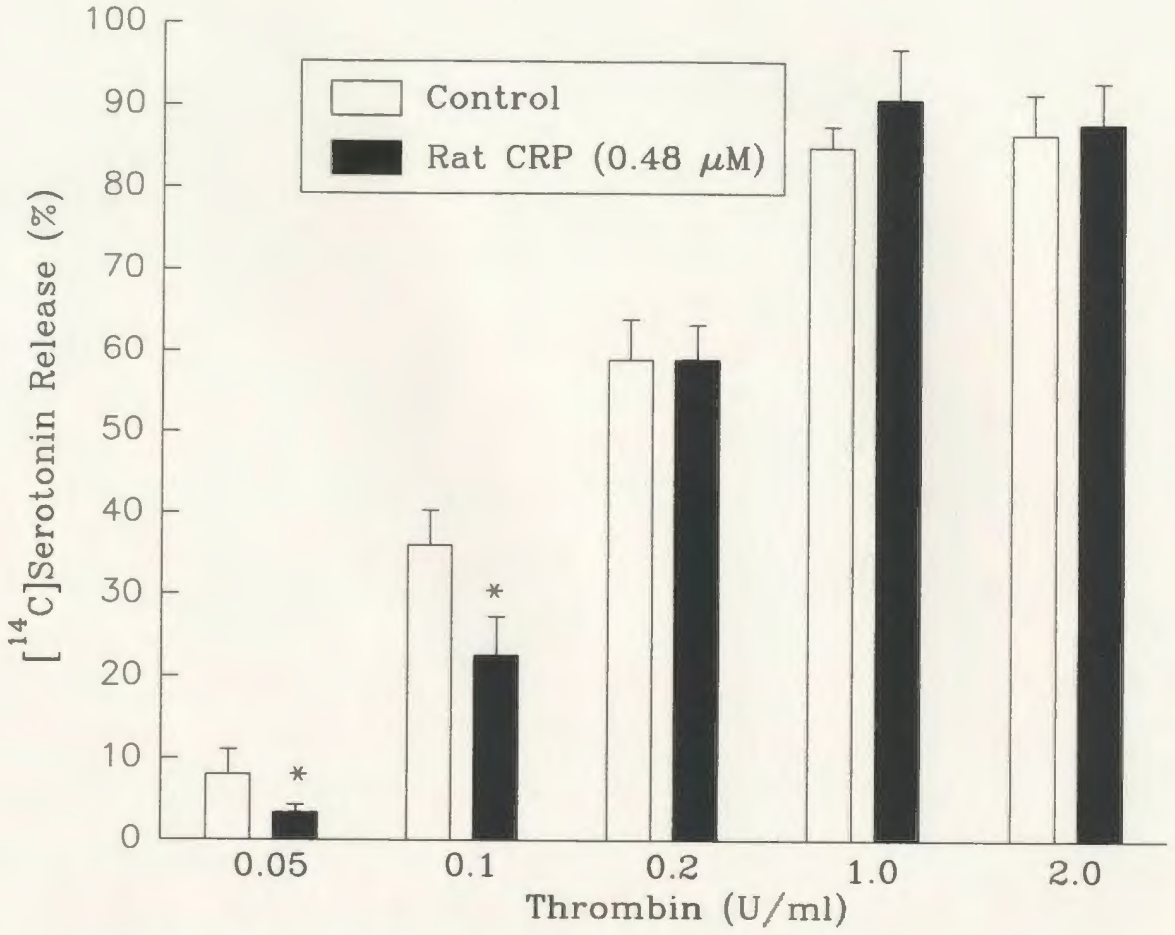


Fig. 16. **Effect of rat CRP on platelet [^{14}C]serotonin secretion induced by increasing concentrations of thrombin.** Washed platelets (4 x 10^8 platelets/ml) were pre-labelled with [^{14}C]serotonin prior to stimulation with various concentrations of thrombin (0.05-2 U/ml). Platelets, pre-incubated in the absence or presence of rat CRP (0.48 μM) for 15 min, were allowed to aggregate for exactly 3.0 min after adding thrombin, and reactions were stopped for determination of [^{14}C]serotonin secretion as described in Fig. 14. Results are means $\pm$ S.E. of 4 separate experiments. * Differences are statistically significant ($p<0.05$) by the students T-test as compared to control values obtained under similar conditions but in absence of rat CRP.

The effect of rat CRP (0.48 μM) on [^{14}C]serotonin secretion by 0.1 U/ml thrombin was also studied as a function of time (Fig. 17). The results of this experiment indicated that the secretion of [^{14}C]serotonin was complete within 1 min after the addition of thrombin to the platelet suspension and this secretion was inhibited by rat CRP.

C. Effect of Rat CRP on PC-PLC Activity in Intact Labelled Platelets, Platelet Sonicates and on PC-PLC Activity from *C. welchii*

The effect of rat CRP on PC-derived DAG from [^{3}H]myristic acid-labelled platelets is shown in Fig. 18. Rat CRP (0.48 μM) significantly ($p<0.05$) inhibited the formation of [^{3}H]DAG by 55 and 70 % when platelets were stimulated by either high (2 U/ml) or low (0.05 U/ml) concentrations of thrombin, respectively. Likewise, rat CRP reduced the formation of [^{14}C]phosphorylcholine, by 40%, from [N-*methyl*-^{14}C]lyso-PAF-labelled platelets stimulated by 2 U/ml thrombin (Fig. 19).

The effect of rat CRP on platelet sonicate PC-PLC activity was examined. The formation of [^{3}H]phosphorylcholine and [^{3}H]DAG by the action of PC-PLC, from platelet sonicates, on exogenous [^{3}H-choline]DPPC or [2-^{3}H palmitoyl]DPPC was inhibited by rat CRP (0.4-2.4 μM) in a dose-dependent manner (Fig. 20A and B).

The effect of rat CRP on the PC-PLC activity in platelet sonicates fractionated

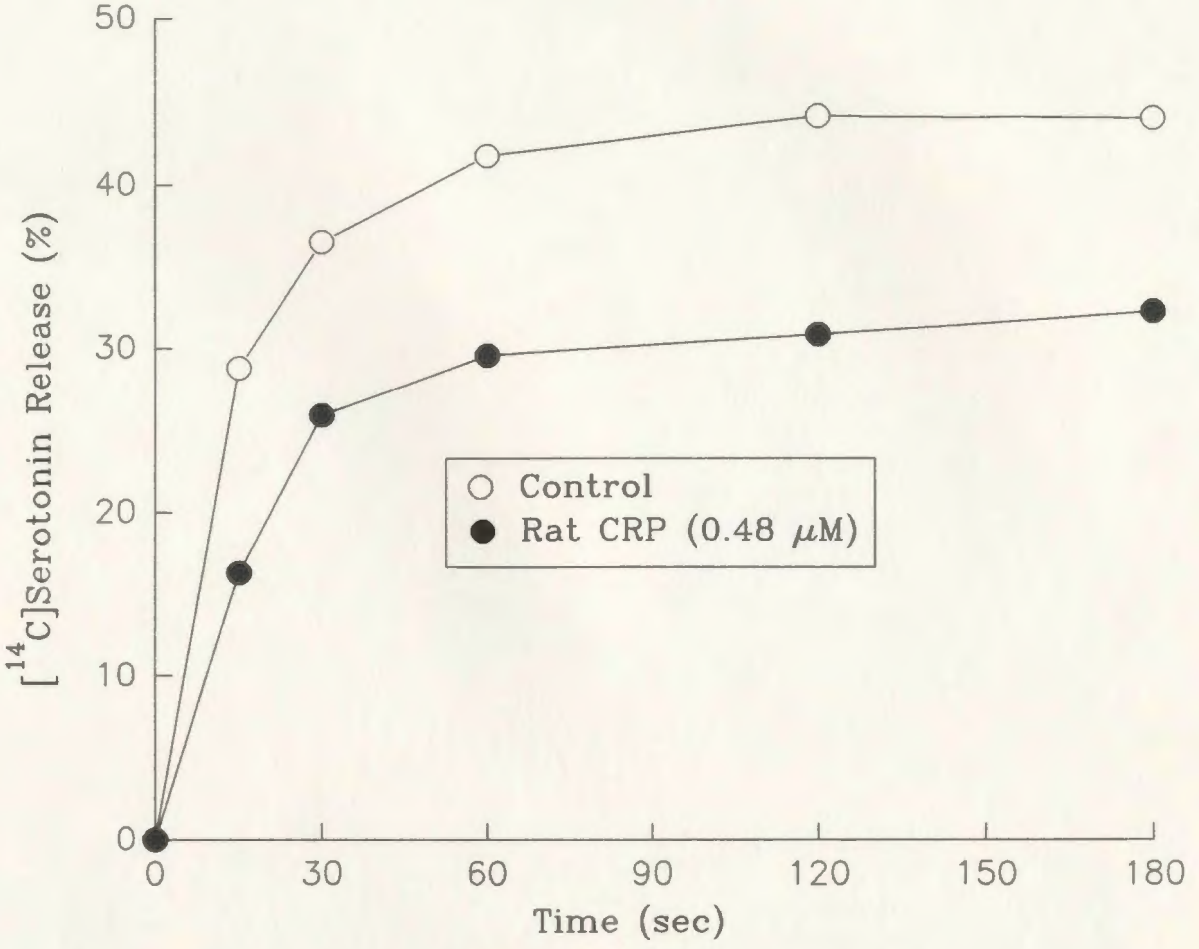


Fig. 17. **Effect of rat CRP on the secretion of [^{14}C]serotonin induced by thrombin as a function of time.** Washed platelets (4 x 10^8 platelets/ml), pre-labelled with [^{14}C]serotonin, were incubated in absence (○) or presence of rat CRP (0.48 μM; ●) for 15 min, prior to stimulation with thrombin (0.1 U/ml). Platelets were allowed to aggregate for specific time periods (0-3.0 min.) and then 100 μl of reaction mixture was stopped for determination of [^{14}C]serotonin secretion as described in Fig. 14. Results are means of 3 determinations.

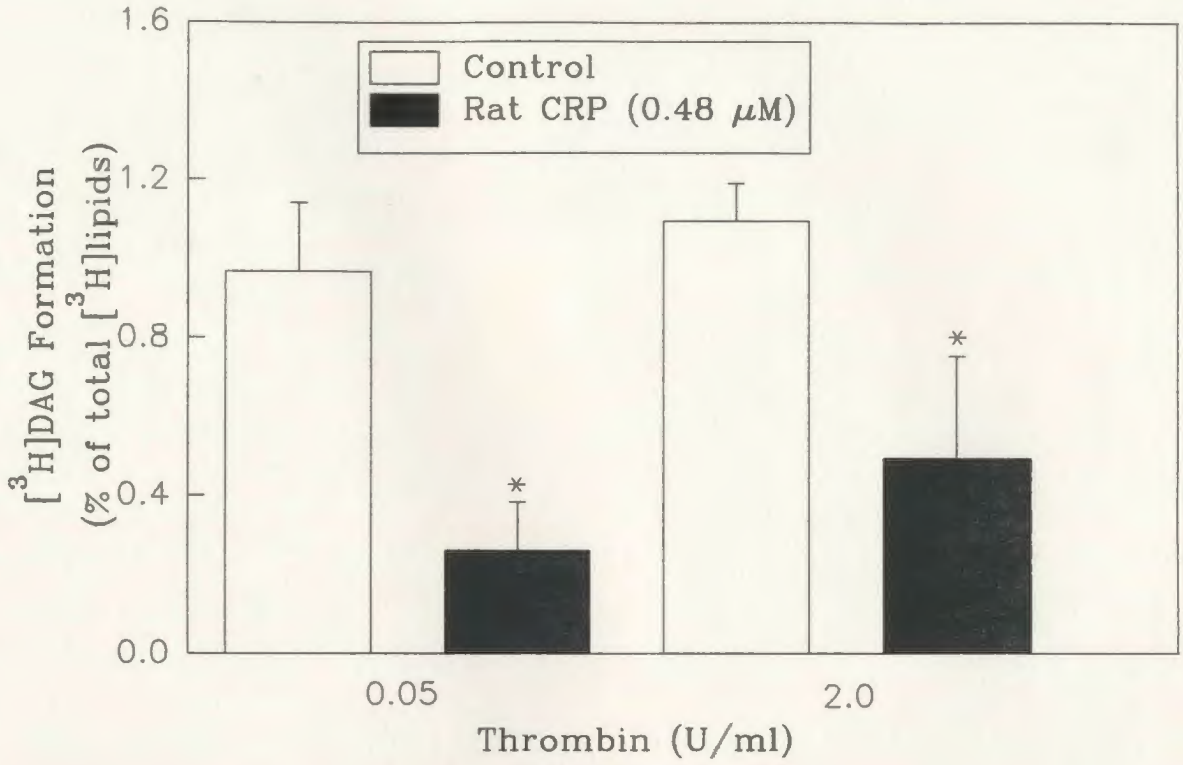


Fig. 18. **Effect of rat CRP on thrombin-induced formation of [^{3}H]DAG from platelets labelled with [^{3}H]myristic acid.** [^{3}H]Myristic acid-labelled platelets were incubated for 15 min either in the absence or presence of rat CRP (0.48 μM). Platelets were activated by the indicated amount of thrombin for 3 min. The [^{3}H]DAG formation was measured as described in Fig. 11. Each value is the mean $\pm$ S.E. of 4 separate experiments. The individual measurements were expressed as percentages of total labelled phospholipid and basal levels, determined in incubations without addition of thrombin, were subtracted. * Differences are statistically significant ($p < 0.05$) by the students T-test as compared to control values obtained under similar conditions but in absence of rat CRP.

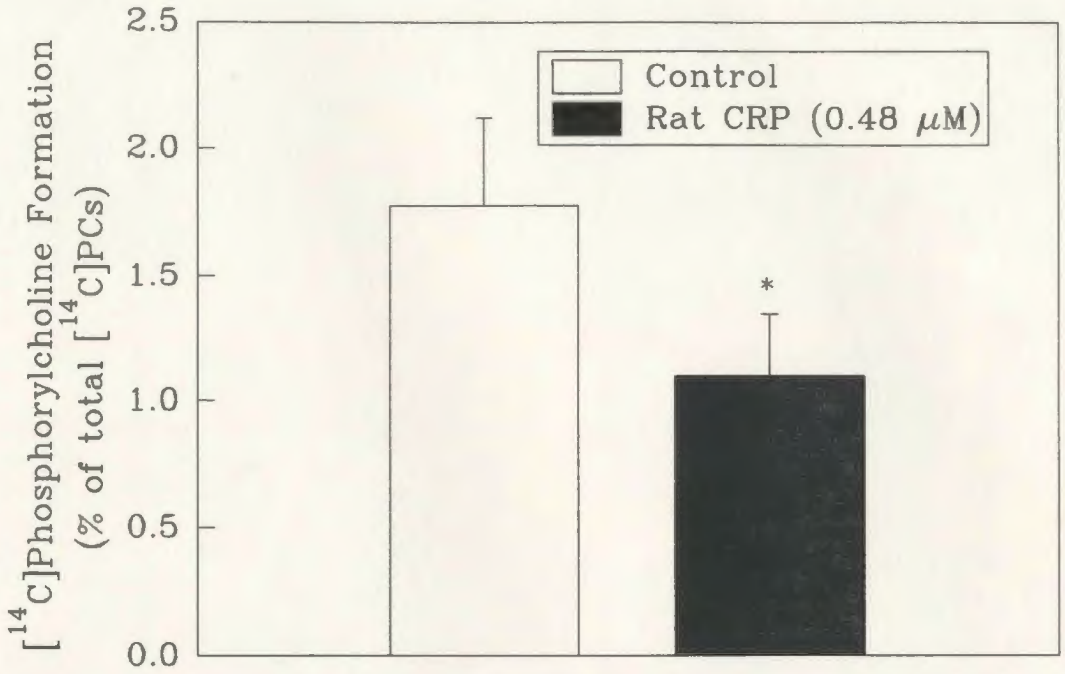
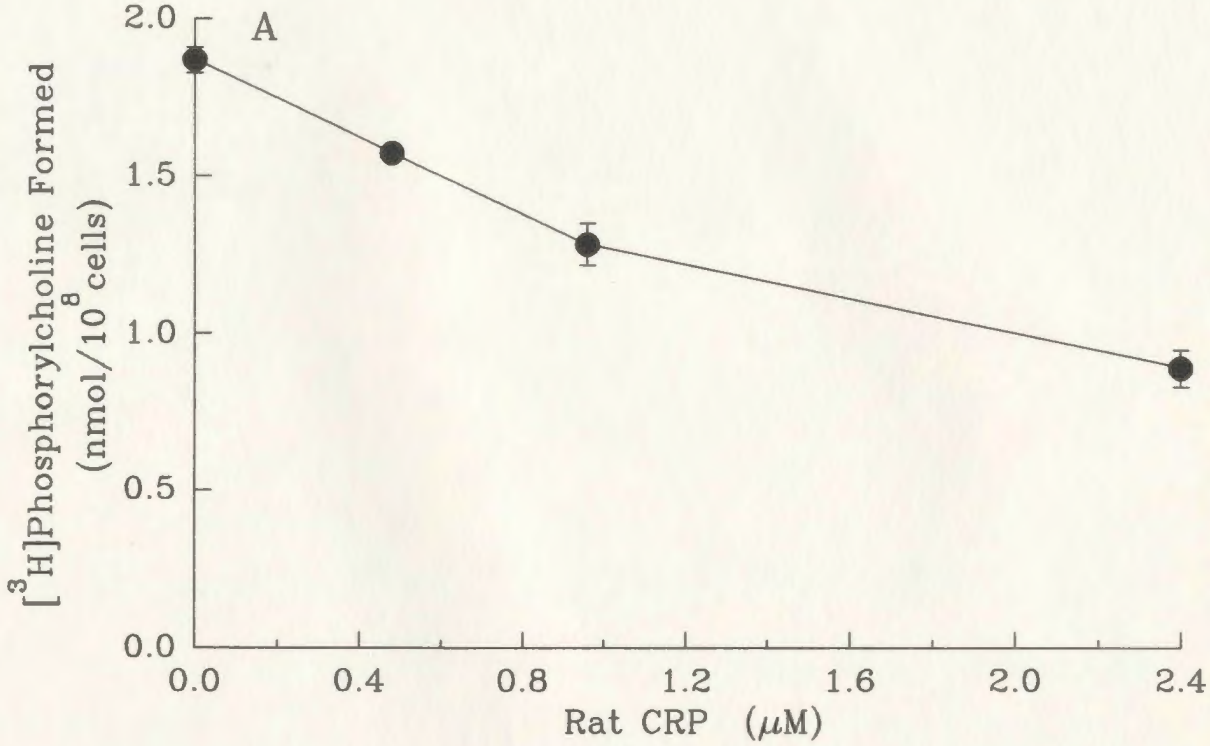
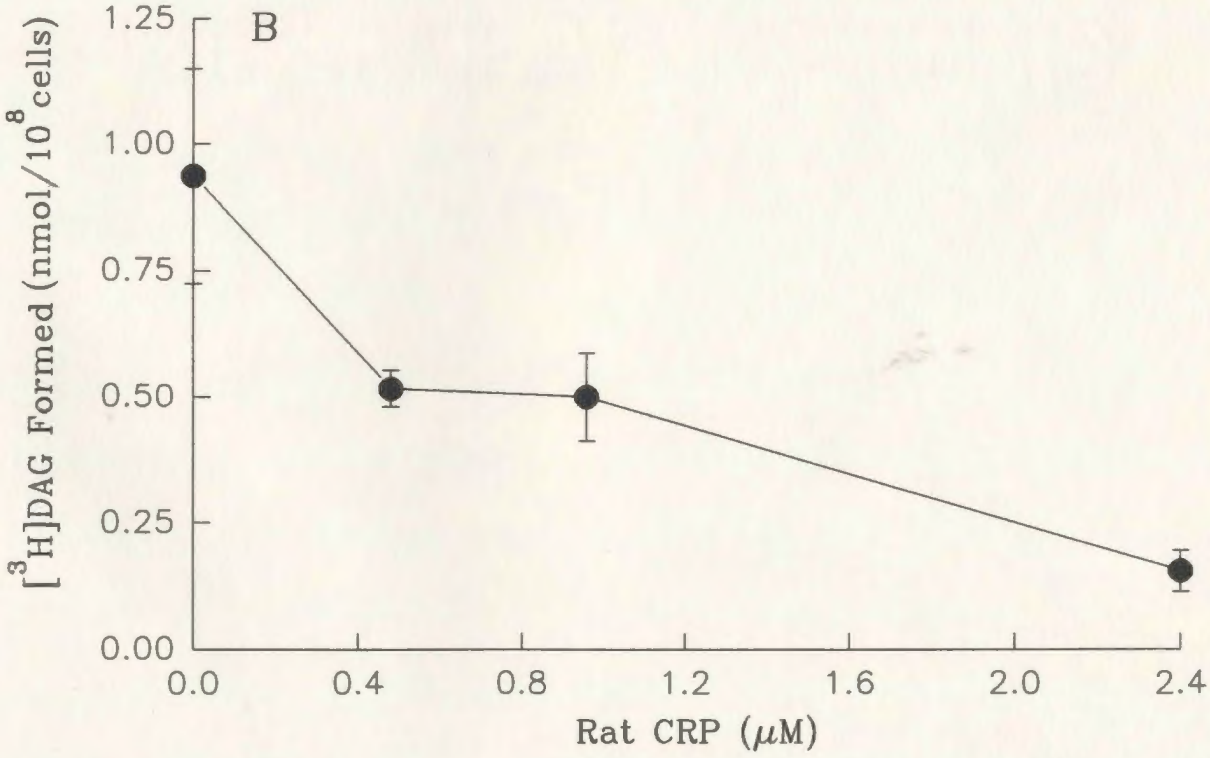


Fig. 19. **Effect of rat CRP on the formation of [^{14}C]phosphorylcholine by thrombin-activated rat platelets labelled with [N-*methyl*-^{14}C]lyso-PAF.**. Platelets labelled with [N-*methyl*-^{14}C]lyso-PAF were incubated in the absence or presence of rat CRP (0.48 μM) for 15 min. Platelets were activated by thrombin (2.0 U/ml) for 3 min. The formation of [^{14}C]phosphorylcholine was measured as described in Fig. 12. Values are mean $\pm$ S.E. from 10 experiments. * Difference is statistically significant ($p<0.05$) by the students T-test as compared to control values obtained under similar conditions but in absence of rat CRP.

Fig. 20. **Effect of rat CRP on PC-PLC in rat platelet sonicates.** Platelet sonicates (4×10^7/ml) were added to standard incubation medium containing A) 18 μM [^{3}H-choline]DPPC or B) [2-^{3}H palmitoyl]DPPC and were carried out in presence of increasing concentrations of rat CRP (0-2.4 μM). After 20 min the reaction was stopped and the amount of either [^{3}H]phosphorylcholine and [^{3}H]DAG formed was determined. The data are mean $\pm$ S.E. of 3 determinations and are representative of 3 experiments.


A
2.0
1.5
1.0
0.5
0.0
[3H]Phosphorylcholine Formed (nmol/10^8 cells)
0.0
0.4
0.8
1.2
1.6
2.0
2.4
Rat CRP (μM)


B
1.25
1.00
0.75
0.50
0.25
0.00
[3H]DAG Formed (nmol/10^8 cells)
0.0
0.4
0.8
1.2
1.6
2.0
2.4
Rat CRP (μM)

by ultracentrifugation at 105,000 x g for 1 h was also examined. Rat CRP (2.4 μM) specifically inhibited the PC-PLC activity in the supernatant ($p<0.05$), while the effect on the particulate form was marginal (Fig. 21).

The effect of rat CRP on PC-PLC activity from *C. welchii* was studied. Fig. 22 shows a dose-dependent inhibition of bacterial PC-PLC activity by rat CRP (0.4-1.6 μM).

D. Effect of Rat CRP on PLA_2 activity in Intact Labelled Platelets, Platelet Sonicates and on PLA_2 from Snake Venom (*Naja naja*)

In order to measure PLA_2 activity in intact rat platelets, platelets were labelled with [^{3}H]arachidonic acid, which provided an endogenously labelled pool of phospholipid (Table 1). The platelets were stimulated with high (2 U/ml) or low (0.05 U/ml) concentrations of thrombin, which resulted in the formation of free [^{3}H]arachidonic acid, predominantly by hydrolysis of PC (Fig. 23).

The effect of rat CRP on the formation of [^{3}H]arachidonic acid and its metabolites from thrombin-stimulated platelets, labelled with [^{3}H]arachidonic acid, was examined. The stimulation of platelets by thrombin (0.05 and 2 U/ml) resulted in the formation of [^{3}H]arachidonic acid and its metabolites, HHT, HETE, and thromboxane B_2 (from thromboxane A_2), formed by the cycloxygenase or the lipoxygenase pathways (Fig. 24). In the presence of rat CRP (0.48 μM) there was no inhibition of the formation of [^{3}H]arachidonic acid or its metabolites (Fig. 24). This

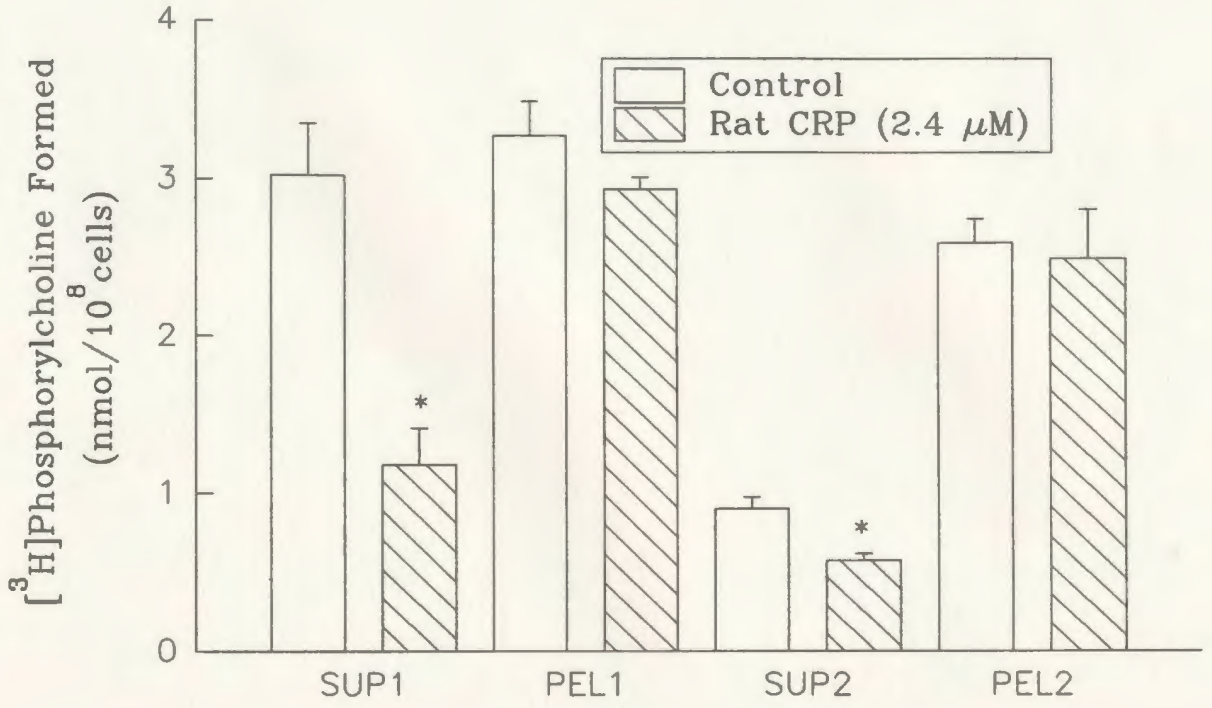


Fig. 21. **Effect of rat CRP on PC-PLC activity in particulate and supernatant fractions of rat platelet sonicates.** Platelet sonicates (5 x 10^7/ml) were centrifuged at 105,000 x g for 1 h to produce a supernatant (SUP1) and pellet (PEL1). The re-suspended particulate fraction (PEL1) was re-centrifuged (105,000 x g) for 1 h to produce a supernatant (SUP2) and pellet (PEL2), which was re-suspended. The supernatant and particulate fractions (40 μl of each) were assayed for PC-PLC activity in absence or presence of rat CRP (2.4 μM). PC-PLC assays were performed using [^{3}H-choline]DPPC as substrate as described in Fig. 20. Values are mean $\pm$ S.E. of 3 determinations. * Differences are statistically significant ($p < 0.05$) by the students T-test as compared to control values obtained under similar conditions but in absence of rat CRP.

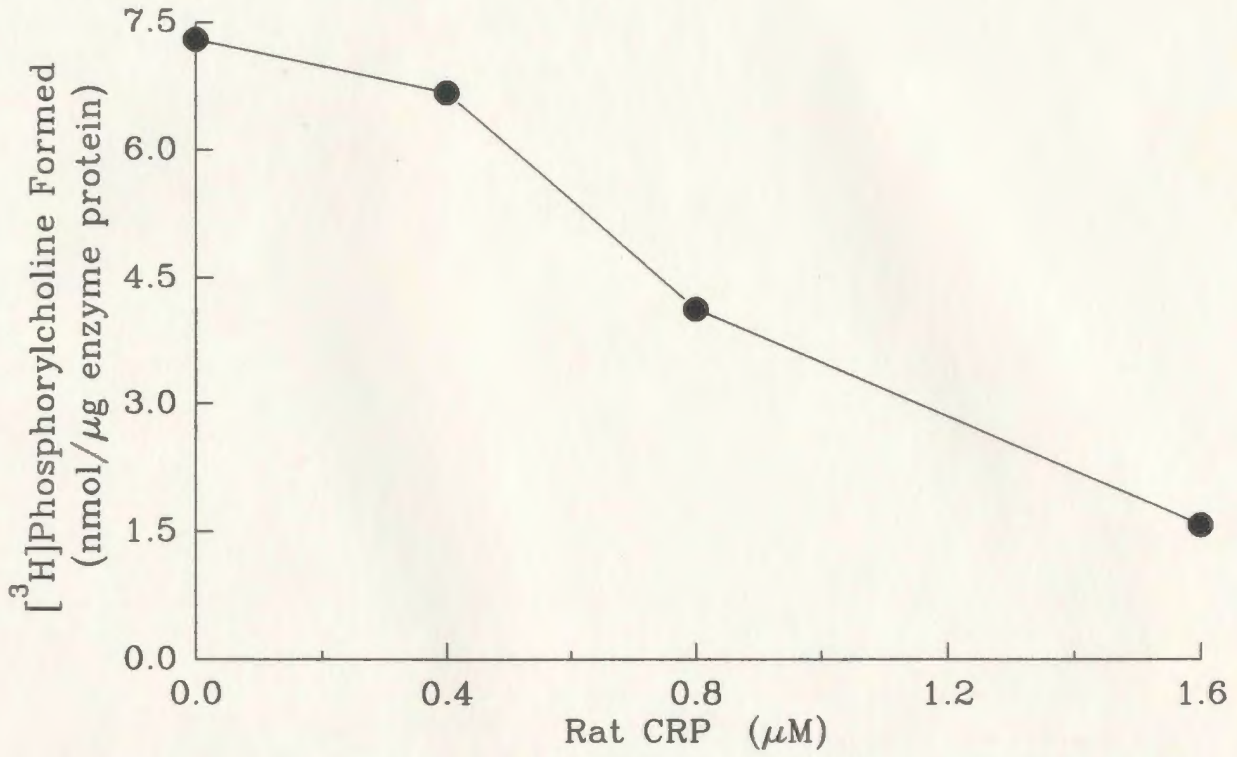


Fig. 22. **Effect of rat CRP on bacterial PC-PLC activity.** Bacterial PC-PLC assays were performed with 0.02 U/ml of enzyme using 150 μM [^{3}H-choline]DPPC as substrate, in the presence of increasing concentrations of rat CRP (0-1.6 μM) as described in Methods and Materials (section VIII.A.). The data are mean of duplicate determinations and are representative of at least 6 experiments.

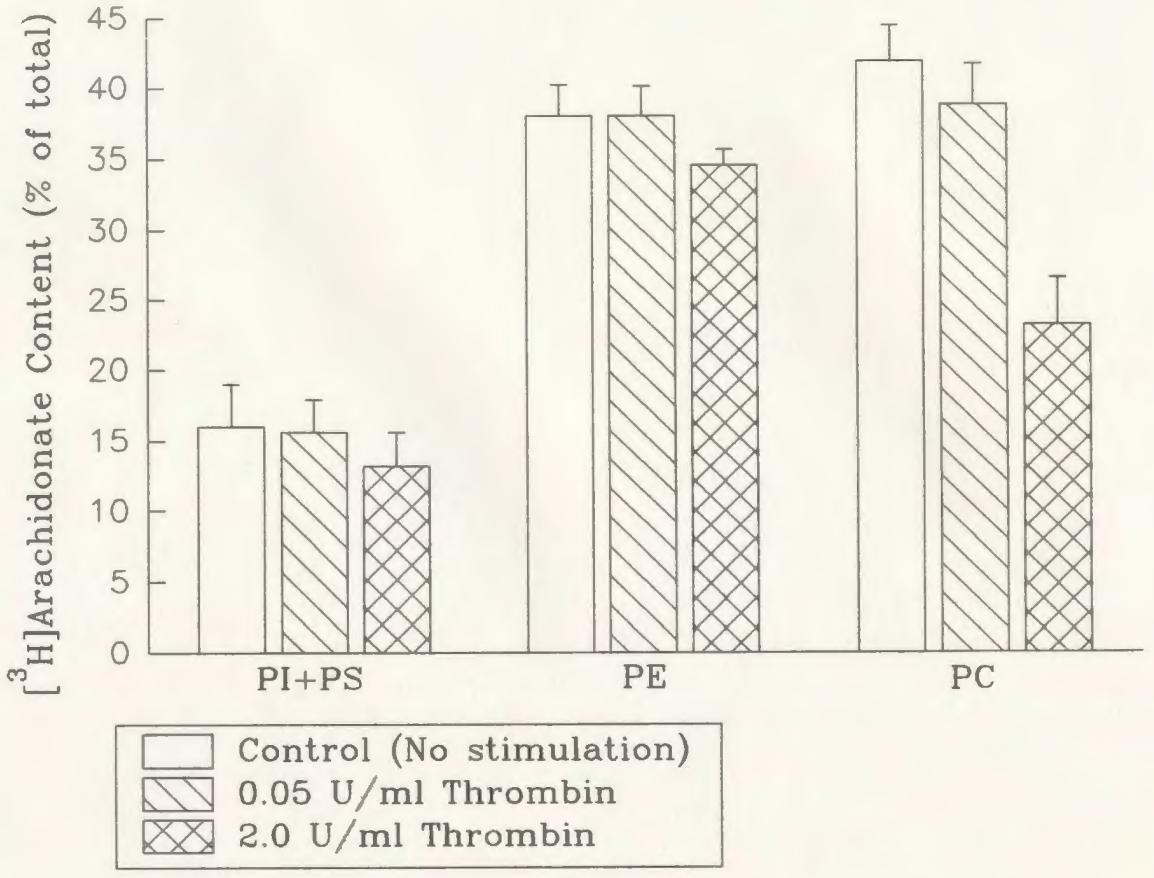
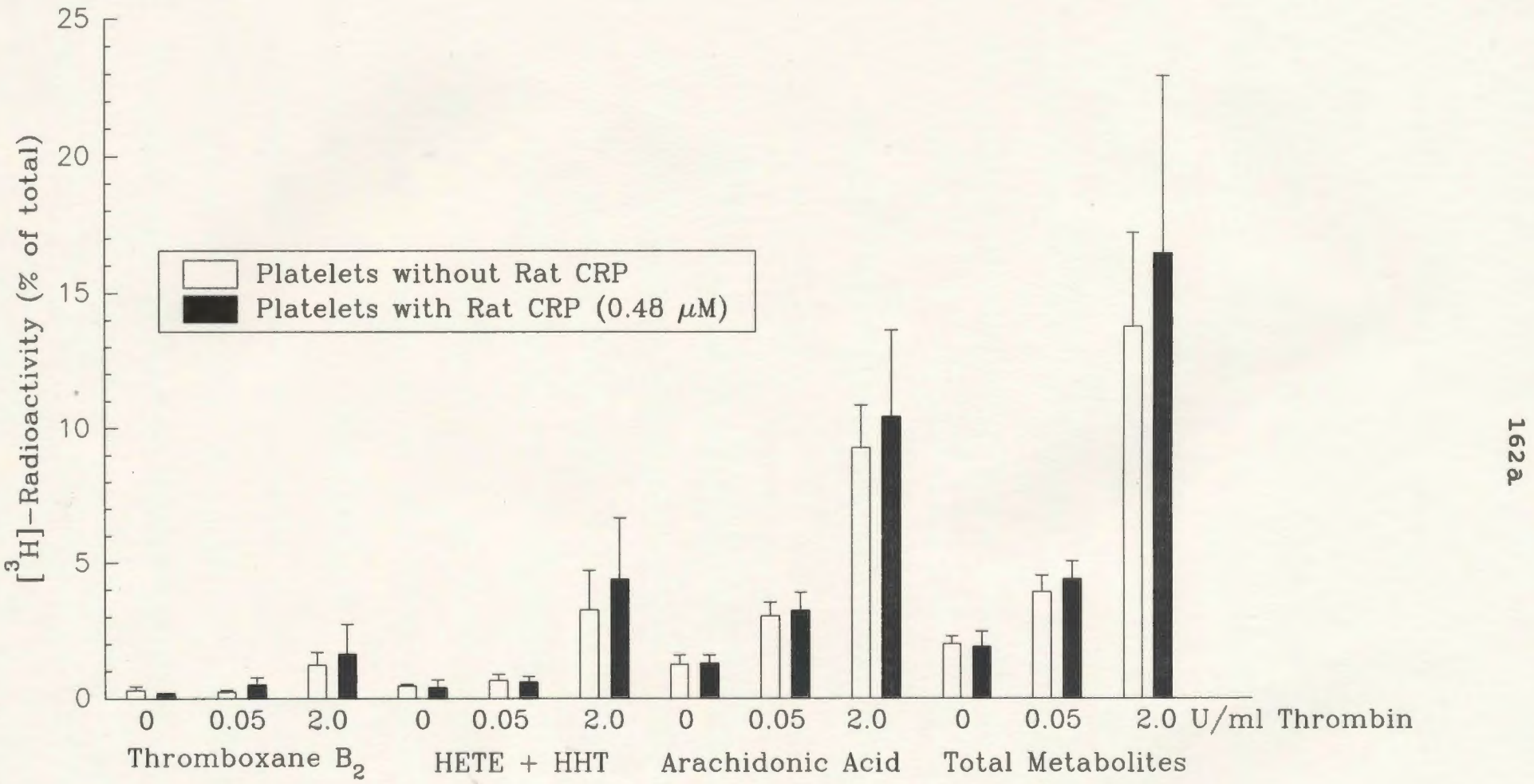


Fig. 23. **Effect of thrombin on the release of [^{3}H]arachidonic acid from platelet phospholipid labelled with [^{3}H]arachidonic acid.** Platelets (5 x 10^8/ml), labelled with [^{3}H]arachidonic acid, were incubated in the absence or presence of thrombin (0.05 U/ml or 2 U/ml) for 15 min. Reaction was stopped by adding 3.75 vol. of chloroform/methanol (1/2) and extracted (Bligh and Dyer, 1959). The organic layer of extracted samples were applied to TLC and phospholipids were separated as described in Methods and Materials (Section IV.C.2.). The radioactivity associated with each lipid group was expressed as a percentage of total radioactivity in that lane. Results are mean $\pm$ S.E. for 4 experiments.

Fig. 24. **Effect of rat CRP on thrombin-stimulated formation of [^{3}H]arachidonic acid metabolites by platelets labelled with [^{3}H]arachidonic acid.** Platelets ($5x10^8$/ml) labelled with [^{3}H]arachidonic acid were incubated in the absence or presence of rat CRP (0.48 μM) for 15 min prior to the addition of indicated amount of thrombin. Platelets were activated for 5 min before stopping reactions and extracting the lipids, which were analyzed by TLC (section IV.C.3.). The radioactivity associated with arachidonic acid and its metabolites (thromboxane B_2, HETE, HHT) were counted and is expressed as percent of total labelled lipid. Results are mean $\pm$ S.E. from 4 different experiments.


25
20
15
10
5
0
[3H]-Radioactivity (% of total)
Platelets without Rat CRP
Platelets with Rat CRP (0.48 μM)
0
0.05
2.0
0
0.05
2.0
0
0.05
2.0
0
0.05
2.0 U/ml Thrombin
Thromboxane B2
HETE + HHT
Arachidonic Acid
Total Metabolites

indicated that rat CRP inhibited neither PLA_2 activity nor the subsequent metabolism of arachidonic acid by lipoxygenase or cycloxygenase.

The effect of rat CRP on PLA_2 activity in sonicated rat platelets was studied. Concentrations of rat CRP ranging from 0.8-3.2 μM did not inhibit the PLA_2 activity (Fig. 25).

The effect of rat CRP on PLA_2 from snake venom (*Naja naja*) was also examined. However, rat CRP at concentrations ranging from 0.4-3.2 μM was found to have no effect on the enzyme activity (Fig. 26).

E. Effect of Rat CRP on PI-PLC Activity in Intact Labelled Platelets and Platelet Sonicates

The effect of rat CRP on thrombin-induced PI-PLC activity was examined using platelets labelled with [^{3}H]inositol. When platelets were stimulated by either 0.05 or 2 U/ml thrombin for 3 min it resulted in dose-dependent formation of [^{3}H]inositol phosphates, including [^{3}H]inositol-1-phosphate, [^{3}H]inositol-1,4-biphosphate and [^{3}H]IP_3 (Fig. 27A-D). However, rat CRP (0.48 μM) did not alter the formation of either [^{3}H]inositol-1-phosphate, [^{3}H]inositol-1,4-biphosphate or the second messenger, [^{3}H]IP_3, in platelets stimulated by either high or low concentrations of thrombin (Fig. 27 B-D).

The effect of rat CRP on PI-PLC activity in rat platelet sonicates was examined (Fig. 28). Concentrations of rat CRP ranging from 0.8-2.4 μM had no

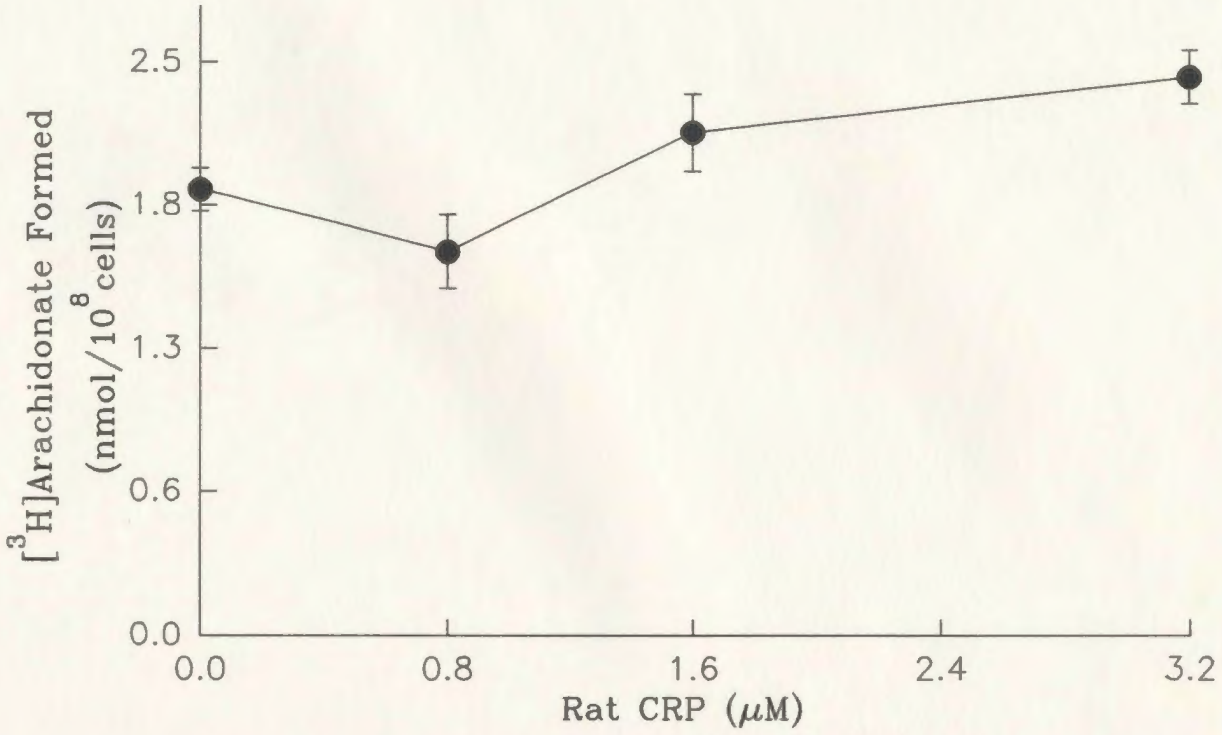


Fig. 25. **Effect of rat CRP on PLA_2 in platelet sonicates.** Platelet sonicate PLA_2 assays were performed using platelet sonicates (8.1×10^7/ml) and [2-^{3}H arachidonyl]PC (50 μM) as the substrate, in the presence of rat CRP (0-3.2 μM), as described in Methods and Materials (section IX.B.). The data are mean $\pm$ S.E. of 3 determinations.

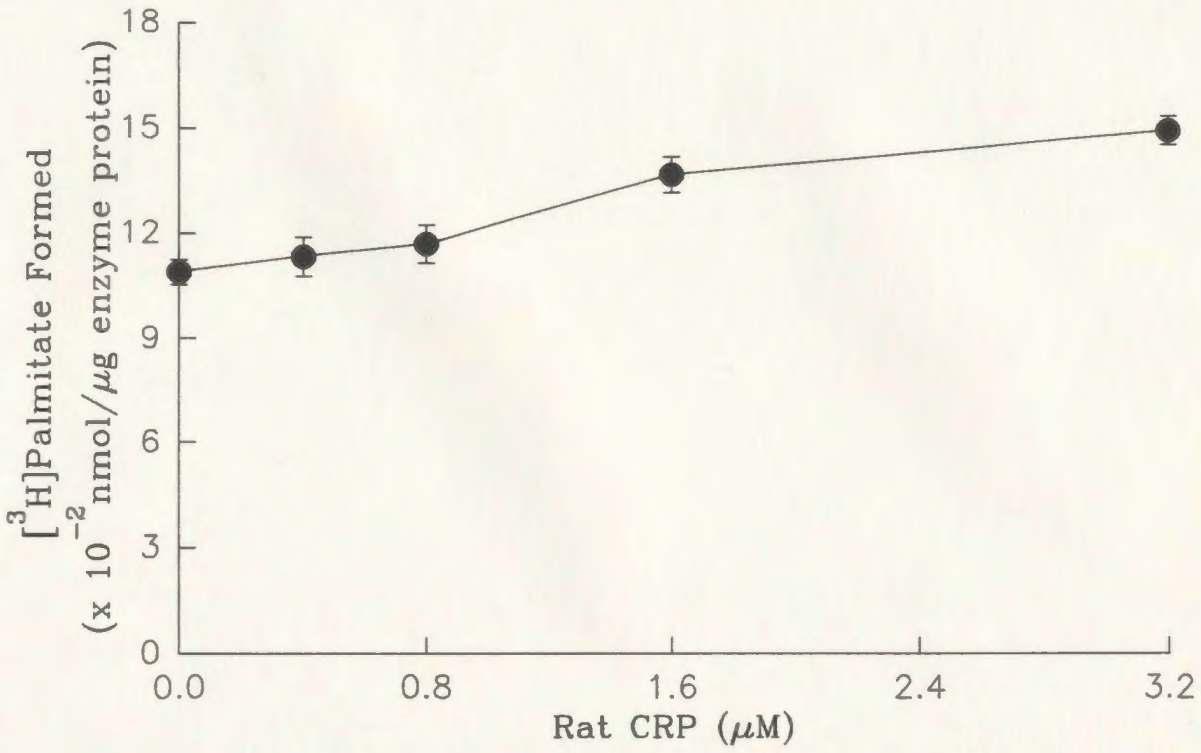
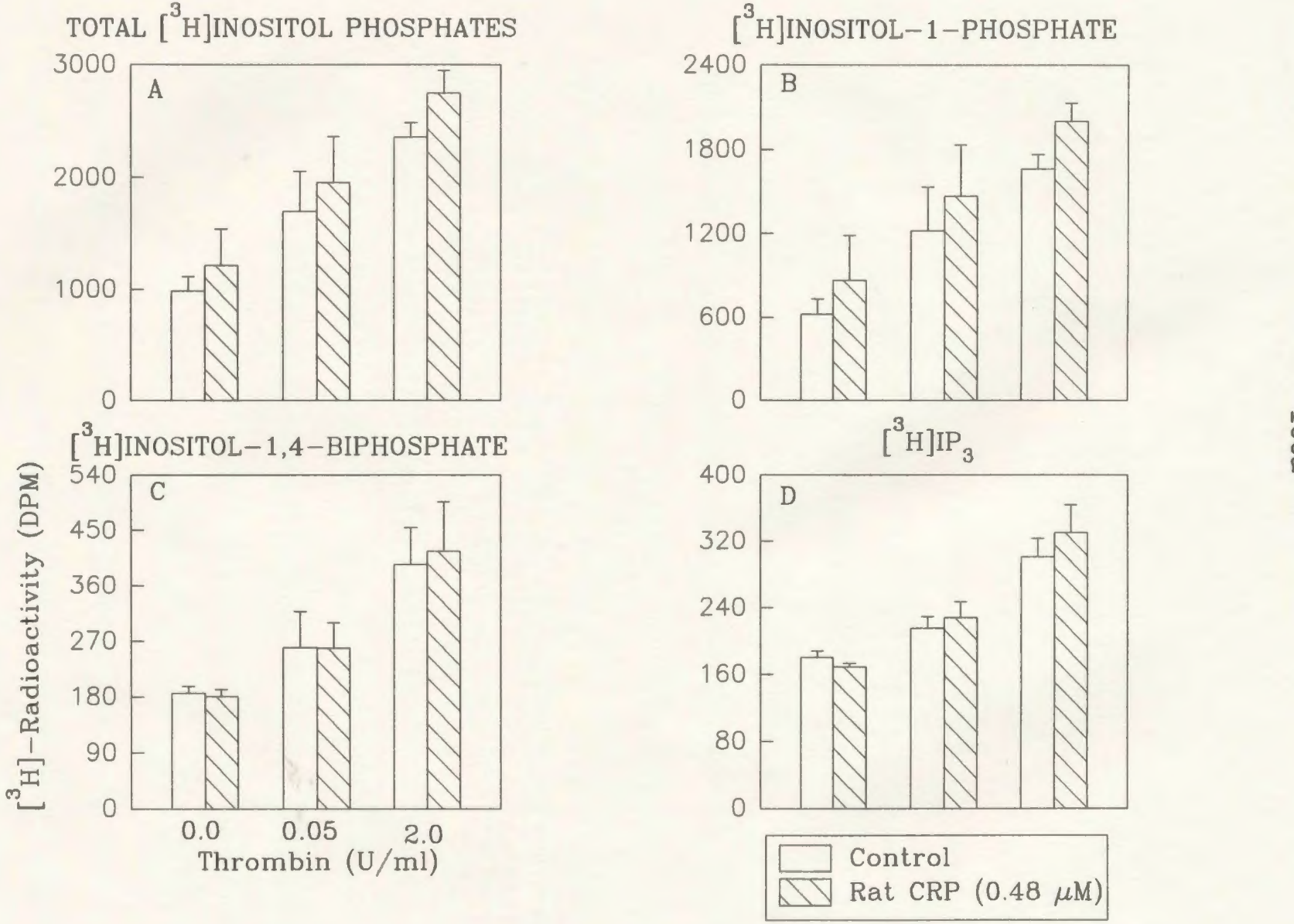


Fig. 26. **Effect of rat CRP on PLA_2 purified from snake venom.** Snake venom PLA_2 assays were performed using 0.02 U/ml of enzyme and 160 μM [2-^{3}H palmitoyl]DPPC as the substrate, in the presence of increasing concentrations of rat CRP (0-3.2 μM), as described in Methods and Materials (section VIII.B.). The data mean $\pm$ S.E. of 3 determinations.

Fig. 27. **Effect of rat CRP on thrombin-stimulated formantion of [^{3}H]inositol phosphates by platelets labelled with myo-[^{3}H]inositol.** Platelets (5 x 10^8/ml), labelled with [^{3}H]inositol, were incubated in the absence or presence of rat CRP (0.48 μM) for 15 min. Platelets were then activated for 3 min by the indicated amount of thrombin. Inositol phosphates were extracted and separated as described in Methods and Materials (section X.C). Values are mean $\pm$ S.E. of 4 separate experiments and are expressed as radioactivity (DPM's) associated with total A) [^{3}H]inositol phosphates, B) [^{3}H]inositol-1-phosphate, C) [^{3}H]inositol-1,4-biphosphate and D) [^{3}H]IP_3.


TOTAL [3H]INOSITOL PHOSPHATES
A
3000
2000
1000
0
[3H]INOSITOL-1-PHOSPHATE
B
2400
1800
1200
600
0
[3H]INOSITOL-1,4-BIPHOSPHATE
C
540
450
360
270
180
90
0
[3H]IP3
D
400
320
240
160
80
0
[3H]-Radioactivity (DPM)
0.0
0.05
2.0
Thrombin (U/ml)
Control
Rat CRP (0.48 μM)

inhibitory effect on the PI-PLC activity in platelet sonicates.

Section III. Discussion

The activation of phospholipases by various agonists has been well documented in the literature (Billah and Anthes, 1990). The interaction of thrombin with its specific receptors on platelet membranes leads to increased turnover of phospholipids by activation of specific phospholipases (Greco and Jamieson, 1991). This turnover of phospholipid in turn mediates the platelet functional responses by the generation of intercellular mediators and second messengers which regulate platelet aggregation and secretion. It is, therefore very likely that rat CRP may inhibit thrombin-induced platelet aggregation and secretion by affecting the platelet phospholipases mediating these responses. Therefore, in this study, the effect of rat CRP on agonist-inducible platelet phospholipases was examined.

The present study demonstrated that rat platelet sonicates hydrolyse [^{3}H-choline]DPPC and [2-^{3}H palmitoyl]DPPC to produce [^{3}H]phosphorylcholine and [^{3}H]DAG, respectively, thus confirming the presence of PC-PLC activity in normal rat platelets. Furthermore, the activity of this enzyme was dependent on time (Fig. 3), substrate (Fig. 4A) and platelet sonicate (Fig. 4B) concentrations. Analysis of the products of PC hydrolysis, revealed phosphorylcholine to be the only labelled metabolite recovered in the aqueous phase. DAG and palmitic acid were the only labelled products in the non-aqueous phase. The formation of DAG was a result of the

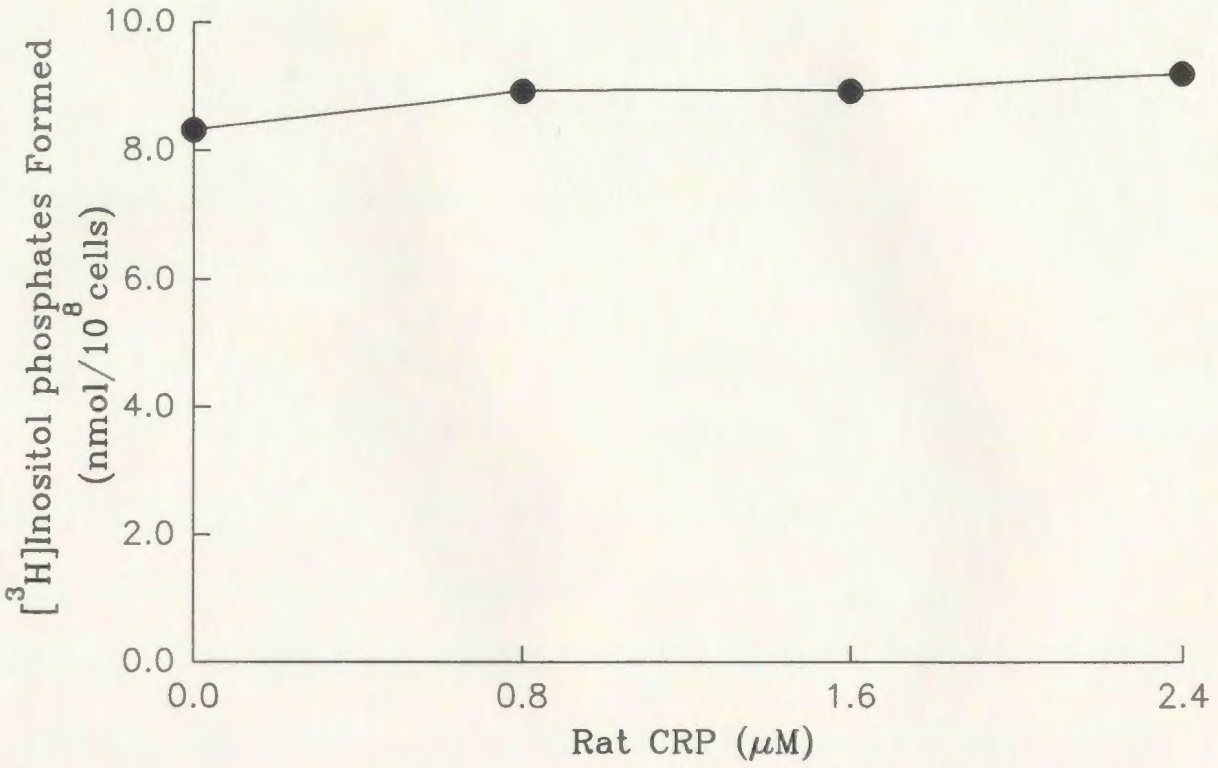


Fig. 28. **Effect of rat CRP on PI-PLC in platelet sonicates.** Platelet sonicate PI-PLC assays were performed using platelet sonicates (2.5 x 10^7/ml) and phosphatidyl[^{3}H]inositol/PE (16 μM; 1:0.4 molar ratio) as the substrate, in presence of rat CRP (0-2.4 μM). Reactions were allowed to proceed for 3 min and were then stopped and extracted as described in Methods and Materials (section IX.C.). The data are mean $\pm$ S.E. of 3 determinations.

action of PC-PLC while palmitic acid was formed by PLA_2 activity in the platelet sonicates (Hayakawa et al., 1988). There was no choline and PA formation, or choline kinase activity in the platelet sonicates. This indicates that PC-PLC activity, and not PC-PLD, was responsible for the generation of phosphorylcholine and DAG. The PC-PLC activity in platelet sonicates was calcium-dependent, and was active over a wide range of calcium concentrations (25 nM - 25 mM) (Fig. 5). This may suggest a role for this enzyme both in the platelet cytosol, where calcium levels are at 0.1 to 3 μM, and maybe outside the platelet where calcium levels are above 1 mM. In addition to rat platelet sonicates, human and rabbit platelet sonicates also showed PC-PLC activity, but the activity was less than 25% of that found in rat platelets. The low activity of PC-PLC activity in rabbit or human platelets may explain why it is not often detected in intact platelets from these species.

Platelet sonicates when fractionated by ultracentrifugation resulted in a pellet and a supernatant fraction. The PC-PLC in the supernatant was designated as the soluble form, while, the PC-PLC in the re-suspended pellet was designated as the particulate form of PC-PLC. Both fractions contained almost equal PC-PLC activity (Fig. 8) which was inhibited by 2 mM EDTA (Fig. 9). The similarities in calcium requirement and substrate specificity for the two forms could indicate a common cellular origin of the enzyme.

The effect of pH on the PC-PLC activity in platelet sonicates, and of the particulate and soluble forms of PC-PLC was determined (Fig. 10). The PC-PLC in

all three cases was found to be optimally active between pH 7.2 and 7.6. It has been reported that PLC with broad substrate specificity exist in lysosomes isolated from rat liver (Matsuzawa and Hostetler, 1978). This lysosomal enzyme was found to be optimally active below pH 5, and was inhibited by divalent cations. It is, therefore, unlikely that the PC-PLC activity associated with platelet sonicates is of lysosomal origin considering its pH optimum and calcium-dependent activity.

Membrane-bound PC-PLD activity has been detected in most rat tissues in the presence of certain detergents (Chalifa et al., 1990; Martin, 1988; Qian and Drewes, 1990). Deoxycholate and β-octylglucoside have been used to measure PC-PLC activity (Anwer et al., 1988; Matozaki and Williams, 1989). The effect of deoxycholate, Triton X-100, and oleate on PC-PLC activity was, therefore, examined (Fig. 7). Of the 3 detergents used in this study only 0.01% deoxycholate and 0.01% Triton X-100 increased the PC-PLC activity. These detergents appeared to have their greatest stimulatory effect on the particulate form of PC-PLC (Fig. 9). The activity of the soluble form of PC-PLC was only moderately increased by 0.01% deoxycholate but was inhibited by 0.01% Triton X-100. No PC-PLD activity was detected, in the platelet sonicates, in the presence of these detergents.

Although there have been several reports of thrombin-induced stimulation of PLD in platelets, there has been no previous report describing the effect of thrombin on rat platelet PC-PLC activity. The results showing a time-dependent increase in DAG- and phosphorylcholine-formation from rat platelets stimulated by thrombin

indicate that the rat platelet PC-PLC is agonist-inducible (Fig. 11-13). Furthermore, the activity of this enzyme requires the presence of thrombin and is not active in unstimulated resting platelets. A number of studies have indicated the possibility of a role for PC-PLC activity in platelets. In a recent study, using platelets from spontaneously hypertensive rats, the generation of PC-derived DAG required for PC synthesis was hypothesised to arise from increased PC-PLC activity in rat platelets (Remmal et al., 1988). It has also been reported that DAG generated by the action of bacterial (*C. welchii*) phospholipase C on platelet plasma membrane PC, resulted in the activation of PKC (Anwer et al., 1988). This PKC is known to phosphorylate a 47 kDa protein involved in platelet responses. This indicates that the generation of PC-derived DAG by PLC can precede the onset of platelet activation and aggregation. A more recent study, in support of this claim, has shown that stimulation of human platelets by HDL_3 resulting in generation of DAG from PC and activation of PKC (Nazih et al., 1990). It is possible that the activation of rat platelet PC-PLC by thrombin is involved in platelet signal transduction pathways.

It has been reported that in a number of tissues, agonist-induced generation of DAG occurs not only from PI hydrolysis but also through the hydrolysis of PC following the activation of PC-PLC or PLD (Billah and Anthes, 1990; Exton, 1990). PI hydrolysis and DAG formation are the earliest signalling events in thrombin-stimulated platelets and DAG is believed to mediate primary (reversible) and secondary (irreversible) aggregation and secretion (Greco and Jamieson, 1991;

Werner et al., 1992). The endogenous production of DAG by either PC or PI is critical in signal transduction presumably through the activation of PKC which is involved in the regulation of cellular responses.

CRP from rat (Chapter 5), rabbit and human have been shown to inhibit platelet aggregation and secretion induced by a variety of agonists (Fiedel and Gewurz, 1976a and b; Fiedel et al., 1977; Vigo, 1985; Kilpatrick and Virella, 1985; Hokama et al., 1984; Filep et al., 1991). In this study the effect of rat CRP on thrombin-activated platelet aggregation and serotonin secretion was examined. The results of this study showed that rat CRP inhibits platelet aggregation and serotonin secretion in a dose-dependent manner when platelets were activated by a low concentrations of thrombin (Figs. 15 and 16). However, rat CRP had little effect on platelet aggregation or serotonin secretion when platelets were activated by high concentration of thrombin. Although previous reports have described the inhibitory effect of CRP on platelet responses, this is the first time the possible role of CRP in the regulation of signalling pathways in platelets has been examined. One previous report does describe the inhibition of platelet PLA_2 by human CRP (Vigo, 1985). The results from this chapter showed that rat CRP inhibited the endogenous formation of both DAG and phosphorylcholine in thrombin-stimulated platelets (Figs. 18 and 19). PC-PLC activity in platelet sonicates and isolated from *C. welchii* were also inhibited by rat CRP (Figs. 21 and 22). Considering that rat CRP inhibited not only the PC-PLC activity in intact platelets but also in platelet sonicates and from bacteria, it is

possible that some CRP-PC interaction on the various membranes may play a key role in the inhibition of this enzyme, since previous studies have shown that rat CRP specifically binds PC-containing liposomes (Nagpurkar et al., 1983). In the PC-PLC assays no formation of choline was observed, consistent with the observation showing the absence of PLD activity in rat platelet sonicates.

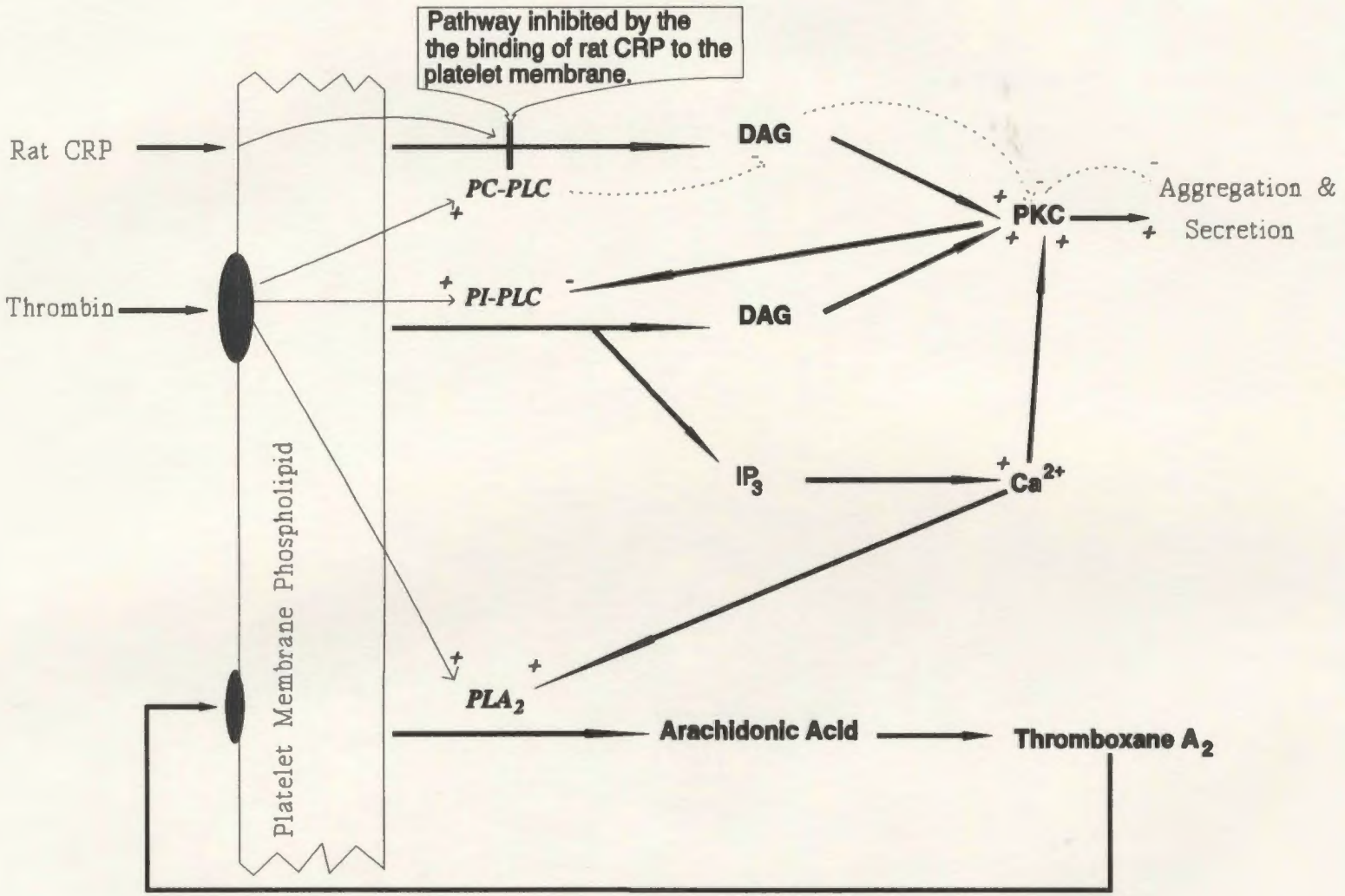
The inhibitory effect of rat CRP was specific to PC-PLC since rat CRP did not inhibit PLA_2 or PI-PLC in platelet sonicates or thrombin-activated platelets. This result with PLA_2 is surprising given the known inhibitory effect of human CRP on PLA_2 activity (Vigo, 1985). It is likely that this is due to the significant differences known to exist between human and rat CRP (Saxena et al., 1985; Rassouli et al., 1992).

The production of DAG (from either PC or PI) may be crucial to the agonist-induced platelet responses (Werner et al., 1991a and b). If DAG is acting as a common and necessary second messenger for the control of cellular responses leading to platelet aggregation and secretion, then modulation of DAG would be an important target for the action of inhibitors of platelet aggregation and secretion. Therefore, platelet inhibitors could prevent aggregation by directly or indirectly inhibiting agonist-induced stimulation of DAG production, which would result in the prevention of the PKC activation required for platelet aggregation and secretion (Werner et al., 1991b). Based on the results showing rat CRP-mediated inhibition of thrombin-induced platelet aggregation, serotonin secretion and specific inhibition of platelet PC-

PLC, it is possible that the inhibition of thrombin-induced platelet aggregation and secretion may be the result of specific inhibition of platelet PC-PLC by rat CRP. Inhibition of PC-PLC activity in platelets by rat CRP in the presence of thrombin would lead to a decrease in the production of PC-derived DAG, which would result in reduced platelet aggregation and serotonin secretion (Fig. 29).

It is significant that rat CRP-mediated inhibition of PC-PLC activity in platelets has been confirmed by examining the effect of rat CRP on PC-PLC activity in platelet sonicates and from *C. welchii*. Since both CRP and PC-PLC enzyme recognise the phosphorylcholine-moiety of PC, recognition of a common substrate might be the key factor in determining the observed specificity of the rat CRP inhibitory action on PC-PLC activity (Fig. 30). By this mechanism rat CRP may bind, via its phosphorylcholine-binding site, to the platelet membrane PC and thus prevent access of PC-PLC to phospholipid. The operation of this mechanism would require that PC-PLC gain access to the PC in the outer leaflet of the platelet plasma membrane. If during platelet activation PC-PLC is situated on the inner leaflet of the platelet plasma membrane, then transmembrane movement of PC (flip-flop) from the outer surface of the platelet plasma membrane should provide PC to the enzyme. Therefore, the binding of rat CRP to PC, which in normally enriched on the outer leaflet of the plasma membrane, may limit the transmembrane movement of PC. This process will limit access of the enzyme to PC and therefore inhibit its activity. The operation of this mechanism in not unlikely considering the increased membrane

Fig. 29. **Scheme for the proposed role of rat CRP in the inhibition of DAG formation during thrombin-induced platelet activation.**



..... Chain of events that may occur as the result of inhibition of PC-PLC by rat CRP.

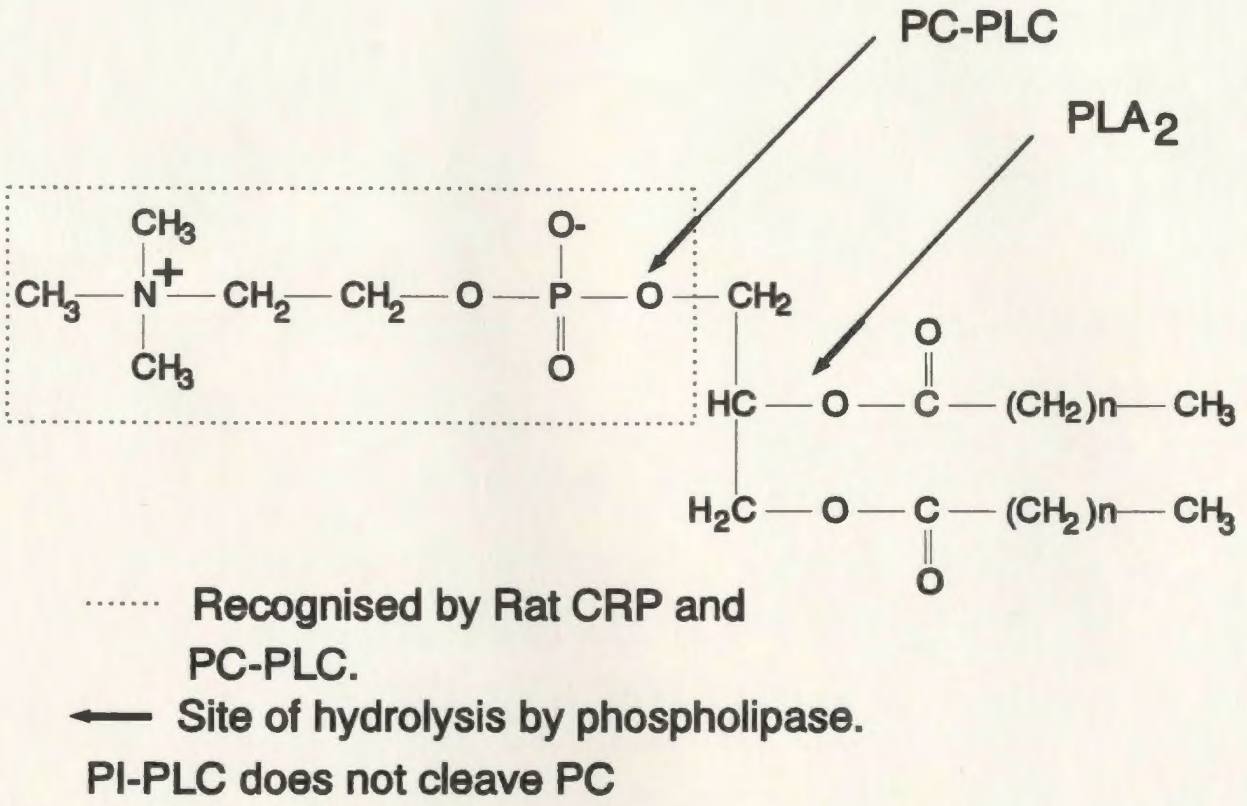
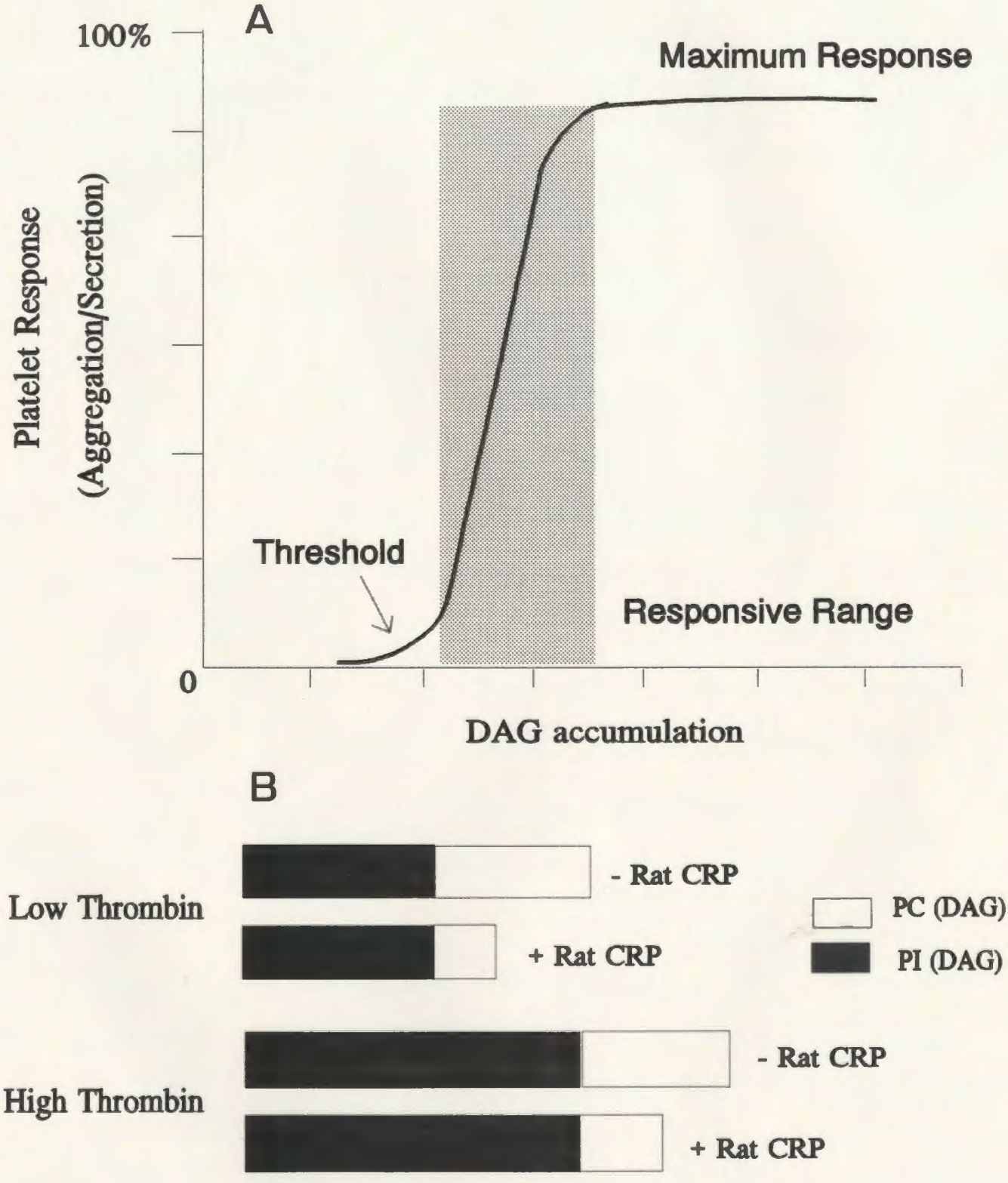


Fig. 30. **Specificity of the interactions of rat CRP and platelet phospholipases with different components of PC structure.**

fluidity and rapid loss of asymmetric distribution of phospholipid, across the platelet plasma membrane during platelet stimulation which increases PC concentrations on the inner leaflet of the plasma membrane (Bevers et al., 1983 and 1990). Another likely mechanism may involve the binding of rat CRP to platelets via some unidentified receptor and, thereby, specifically inactivating or preventing the activation of PC-PLC by thrombin.

Based on the recent report by Werner et al. (1991a), which shows a correlation between DAG levels and platelet aggregation and secretion, the range between threshold DAG levels and those required to induce a maximal response of platelet aggregation or secretion are quite narrow (Fig. 31). DAG levels must reach threshold concentrations in order to perform as an effective second messenger to evoke platelet response. At concentrations of DAG much higher than the responsive range minor changes in DAG concentration apparently will not lead to changes in the platelet response caused by DAG. On this basis, it is likely that the stimulation of platelets by high concentrations of thrombin results in both PI- and PC-derived DAG levels to rise far beyond the responsive range, in which a small reduction in PC-derived DAG levels by rat CRP would not be sufficient to bring about any inhibition in platelet aggregation or serotonin secretion. Furthermore, high concentrations of thrombin result in increased activation of PLA_2, and the consequent formation of arachidonic acid and its metabolites which may augment subsequent platelet responses (Arita et al., 1989). Therefore, in the presence of high concentrations of thrombin,

Fig. 31. **Relationship between inhibition of PC-derived DAG by rat CRP, and platelet aggregation and serotonin secretion.** In a recent report by Werner et al. (48), showing a quantitative correlation between endogenous DAG levels and human platelet aggregation and secretion, it was found that the responsive range between threshold DAG levels and DAG levels required to induce a maximal platelet response was quite narrow (A). When the concentration of DAG was above the responsive range maximum platelet aggregation and secretion occurred and beyond this range minor changes in DAG concentration did not lead to corresponding changes in platelet response. Based on these findings the result of the lack of inhibition of platelet aggregation and secretion by rat CRP (Figs. 15 and 16) when high concentration of thrombin was used can be explained. It is likely that stimulation of platelets by high concentration of thrombin results in the formation of DAG from both PI and PC. This the DAG concentration would be far beyond the responsive range. Therefore, in the presence of high concentration of thrombin the inhibition of PC-PLC by rat CRP would not be sufficient to bring DAG levels within the responsive range (B). In contrast, when low concentration of thrombin was used, the level of DAG formed from both from PI and PC would be within the responsive range, in which a small reduction in the PC-derived DAG by rat CRP would be sufficient inhibit platelet aggregation and secretion.



Adapted from Werner et al. Mol. Phar. 41, 382-386 (1991)

PI-PLC and PLA_2 would generate sufficient levels of appropriate mediators to induce maximum platelet aggregation and serotonin secretion. This may explain why platelet responses were not inhibited by rat CRP when high concentrations of thrombin were used to stimulate the platelets. In contrast, when sub-maximal aggregation or secretion is evoked, a minor decrease in the amount of DAG produced will lead to profound inhibitory effects on the observed response. In the presence of low concentrations of thrombin, which stimulates the production of sub-maximal total DAG levels and, therefore, sub-maximal response, it is possible that the reduction in total DAG levels by rat CRP results in the observed inhibition of platelet aggregation and serotonin secretion. Under these conditions the activity of PLA_2 and production of biologically active eicosanoids is also minimal (Fig. 24).

The binding of rat CRP to specific sites on the platelets was shown in Chapter 6. It was proposed that this binding was a requirement for the inhibition of platelet aggregation (Chapter 5). It is equally possible that rat CRP-mediated inhibition of thrombin-induced platelet aggregation also requires the binding of rat CRP to platelets. The possibility that rat CRP inhibits platelet responses, by preventing the binding of thrombin to its platelet receptors, is unlikely since this would result in decreased activities of both PI-PLC and PLA_2, in addition to PC-PLC activity. Therefore, the rat CRP binding site on rat platelets is most likely unrelated to the thrombin receptors on platelets.

A rather simple mechanism is presented by which rat CRP may inhibit platelet

function, however, it is recognised that the mechanisms of signal transduction in platelets are complex with many potential interrelationships between the various pathways. The relationship of PC-PLC activity to other signal transduction pathways has not been worked out. It is, therefore, not possible to unequivocally attribute the inhibitory effect of rat CRP on platelet aggregation to the inhibited action of PC-PLC activity alone or generalize these observations to explain the inhibitory effects of rabbit or human CRP on platelet aggregation in other species, although rabbit and human CRP have been shown to inhibit the PC-PLC activity in rat platelet sonicates (result not shown). The activation of rat platelet PC-PLC activity by thrombin may be a property unique to this species.

CHAPTER 8

PERSPECTIVE AND FUTURE DIRECTIONS

This thesis has examined the role of rat CRP in the modulation of agonist-induced platelet function. Rat CRP has been shown to specifically inhibit only weak stimuli-induced platelet aggregation and secretion. This inhibitory effect involved a rat CRP-platelet interaction. Further characterization indicated this binding to be specific, saturable and reversible. The possibility of a rat CRP-agonist interaction was also examined. Such interaction, in addition to the rat CRP-platelet interaction, occurred only when PAF was used as an agonist. The binding of rat CRP to PAF was calcium-dependent and involved the phosphorylcholine-binding site on rat CRP. A role for rat CRP in the refractory property of rat platelets to PAF has also been assigned.

The finding of an agonist-inducible PC-PLC in platelets and its likely involvement in the regulation of platelet function through the formation of DAG, led to the studies on the effect of rat CRP on platelet phospholipases. Rat CRP specifically inhibited PC-PLC but not PLA_2 or PI-PLC. It was concluded that if the PC-derived DAG is critical to signal transduction pathways in the regulation of platelet function, then the inhibition of DAG production mediated by PC-PLC is a likely mechanism for the action of rat CRP on platelet aggregation and secretion.

Since the normal concentrations of rat CRP in blood (approximately 0.5-0.6 mg/ml or 4-5 μM) is much greater that the amounts required for maximum inhibition

of platelet aggregation and secretion, it is possible that the inhibitory effect of rat CRP on platelet function may be relevant to events that may take place the animal following injury. Injury, involving damage of endothelium surrounding blood vessels, usually results in the release of platelet stimuli such as ADP, exposure of collagen in the basement membrane, activation of the clotting cascade and formation of thrombin in blood. If the activity of platelet agonists such as thrombin and ADP are left unregulated, such injury could lead to uncontrolled thrombotic events with fatal consequences. Therefore, the existence of control mechanisms for thrombotic events are imperative to the survival of the animal. Rat CRP may be one component by which thrombotic events are controlled.

The binding of rat CRP to platelets may be a prerequisite for the inhibitory effect of rat CRP on platelet function. Furthermore, binding sites for rat CRP present on rat, rabbit and human platelets are not unique for rat CRP, since rabbit CRP competitively inhibited the binding of rat CRP to platelets. It is possible that human CRP, which is structurally very similar to rabbit CRP, would also behave like rabbit CRP with respect to binding to platelets. In connection with this point, both rabbit and human CRP have been shown to inhibit platelet aggregation, indicating that the binding sites on platelets would recognise CRP's from different species and inhibit platelet aggregation.

Although the results presented in this thesis were obtained using rat CRP, rabbit and human CRP were also used in certain experiments involving platelet

aggregation, and platelet phospholipases. The results from these experiments were similar to those obtained with rat CRP with respect to its inhibition of platelet aggregation and platelet sonicate PC-PLC activity (Results not shown). It could, therefore, be argued that in spite of the significant differences (for example: glycosylation, subunit composition, pI, levels in blood, minor amino acid sequence differences) between rat CRP, and human and rabbit CRP, these CRP's may work in the platelets through a common mechanism.

It is interesting to note that the same pathological conditions which give rise to increased PAF concentrations also result in a rise in CRP concentrations. The specific binding of CRP to PAF suggest a relationship between these coincidental events. It is, therefore, not difficult to envision a role for rat CRP as a protective molecule which prevents escalation of the harmful effects of PAF activity in the body during disease. If this is true, then the rat CRP-PAF binding process may not only inhibit PAF-induced platelet function, but also inhibit the effect of PAF on other systems.

The results in this thesis clearly show that the generation of the second messenger DAG from hydrolysis of PC by PC-PLC occurs in rat platelets. The interesting observation that the inhibitory effect of rat CRP is specific to PC-PLC, made in this thesis, may be extended to other blood cells like monocytes, macrophages or neutrophils, that bind to CRP. There have been recent reports describing the involvement of PC-PLC in mediating cellular function in other tissues including certain blood cells (reviewed by Billah and Anthes, 1990; Exton, 1990). It

is possible that the regulation of the function of these cells might be dependent on the generation of DAG through the action of PC-PLC. It is equally conceivable that CRP as a consequence of the binding to these cells may modulate cellular function by a mechanism that involves inhibition of PC-PLC as proposed for rat platelets.

Future Directions

The results of this thesis indicate that rat and rabbit CRP have common specific binding sites on platelets. However, the molecular mechanism by which these binding sites inhibit PC-PLC activity and mediate platelet function remains unknown. It would, therefore, be interesting to further characterize these binding sites by use of specific proteases or phospholipases to identify whether these binding sites involve receptor proteins or lipids. If the binding site appears to be a protein, then isolation of this receptor protein could be carried out. These studies may also aid in establishing the relationship between the binding sites for rat CRP on platelets and PC-PLC activity.

CRP has been shown to bind to cells of the immune system (monocytes, neutrophils, macrophages) and modulate their function. Since these cells may also have PC-PLC activity modulating cellular reactions, it would be interesting to a) confirm/determine the presence of PC-PLC in these cells and b) determine if CRP has any effect on this enzyme in these cells.

The subcellular location of certain enzymes are good indications of their role

in cellular function. Therefore, to further determine the role of PC-PLC in platelet function it would be interesting to determine the sub-cellular location(s) of PC-PLC in the platelet. Based on the results in this thesis it appears that PC-PLC is present as a soluble and also as a membrane bound form in platelets. This may indicate different roles for the two forms. In addition, previous reports have suggested that the release of certain soluble phospholipases occurs during platelet activation (Horigome et al., 1987; Hayakawa et al., 1988). Therefore, it would be interesting to determine that if, as a result of agonist-induced activation of platelets, PC-PLC is released as a soluble enzyme into the extracellular medium. The potential role for the release of this enzyme on platelet function could also be examined.

The presence of an agonist-inducible PC-PLC activity in rat platelets is indicative of a role for this enzyme in regulating platelet functional response. Because of the important role played by DAG in signal transduction mechanisms, it is very possible that this enzyme may play a role in mediation of platelet responses to other agonists besides thrombin. While, evidence for a role of PI hydrolysis in ADP-mediated platelet activation has been controversial, no previous study has examined the possibility of PC-derived DAG as playing a second messenger role during ADP-induced platelet activation. If PC-derived DAG does participate in signal transduction mechanisms stimulated by ADP, then further work may establish an essential role for PC-PLC activity in ADP-induced platelet aggregation. Such findings would revolutionize our current understanding of the mechanisms of platelet activation,

particularly when weak agonists are involved.

It would be important to characterize the mechanisms of activation of PC-PLC by agonists. Because experiments using platelet sonicates indicated that the activity of this enzyme requires calcium, it is possible that an increase in intracellular calcium concentrations may be the stimuli of this enzyme activity in intact platelets. Examining the effect of calcium mobilizing agents, as well as G-protein and PKC activators or inhibitors, on the activity of this enzyme may provide useful clues to its regulation by known pathways of activation. Furthermore, an examination of the DAG species produced as a result of stimulation of platelets may determine the relative importance of PI- and PC-derived DAG in platelet activation.

In any case, the results presented in this thesis have provided some basic information on the role played by CRP in platelet function. Based on this information, the future research directions suggested in this section, when carried out, would eventually clarify the physiological role of CRP in regulation of PC-PLC activity and platelet function.

CHAPTER 9

REFERENCES

Abernethy, T.J., and Avery, O.T. (1941) The occurrence during acute infections of a protein not normally present in the blood. I. Distribution of the reactive protein in patients sera and the effect of calcium ion on the flocculation reaction with the C-polysaccharide of the pneumococcus. *J. Exp. Med.* **73**, 173-182.

Abernethy, T.J., and Francis, T. (1937) Studies on the somatic C-polysaccharide of pneumococcus. I. Cutaneous and serological reactions in pneumonia. *J. Exp. Med.* **65**, 59-73.

Anderson, H.C., and McCarty, M. (1951) The occurrence in the rabbit of an acute phase protein analogous to human C-reactive protein. *J. Exp. Med.* **93**, 25-36.

Anderson, J. K., and Mole, J. E. (1982) Large scale isolation and partial primary structure of human plasma amyloid P-component. *Ann. N. Y. Acad. Sci.* **389**, 216-234.

Anderson, J.K., Stroud, R.M., and Volanakis, J.E. (1978) Studies on the binding specificity of C-reactive protein for phosphorylcholine. *Fed. Proc.* **37**, 1495.

Anthes, J.C., Eckel, S., Siegel, M.I., Egan, R.W. and Billah, M.M. (1989) Phospholipase D in homogenates from HL-60 granulocytes: implications of calcium and G protein control. *Biochem. Biophys. Res. Comm.* **163**, 657-664.

Anwer, K., Wallace, D., Romstedt, K., and Huzoor-Akbar (1988) Human platelet activation by bacterial phospholipase C: mechanism of inhibition by flurazepam. *Thromb. Res.* **49**, 225-239.

Apitz-Castro, R., Jain, M.K., Bartoli, F., Ledezma, E., Ruiz, M.-C., and Salas, R., (1991) Evidence for direct coupling of primary agonist-receptor interaction to the exposure of functional IIb-IIIa complexes in human blood platelets. Results from studies with the antiplatelet compound ajoene. *Biochim. Biophys. Acta* **1094**, 269-280.

Arita, H., Nakano, T., and Hanasaki, K. (1989) Thromboxane A_2: Its generation and role in platelet activation. *Prog. Lipid Res.* **28**, 273-301.

Bach, B.A., Gewurz, H., and Osmand, A.P. (1977) C-reactive protein in the rabbit: Isolation, characterization and binding affinity to phosphocholine. *Immunochemistry* **14**, 215-219.

Balduini, C.L., Sinigaglia, F., Bertolino, G., Bisio, A., Noris, P., and Ascari, E. (1987) Surface mechanisms of platelet adhesion and aggregation. *Haematol.* **72**, 261-268.

Ballou, S.P. and Cleveland, R.P. (1991) Binding of human C-reactive protein to monocytes: analysis by flow cytometry. *Clin. Exp. Immunol.* **84**, 329-335.

Baltz, M.L., De Beer, F.C., Feinstein, A., Munn, E.A., Milstein, C.P., Fletcher, T.C., March, J.F., Taylor, J., Bruton, C., Clamp, J.C., Davies, A.J.S., and Pepys, M.B. (1982) Phylogenetic aspects of C-reactive protein and related proteins. *Ann. N.Y. Acad. Sci.* **389**, 49-69.

Baltz, M.L., De Beer, F.C., Feinstein, A., and Pepys, M.B. (1982) Calcium-dependent aggregation of human serum amyloid P component. *Biochim. Biophys. Acta* **701**, 229-236.

Banga, H.S., Halenda, S.P., and Feinstein, M.B. (1991) Potentiation of arachidonic acid release by phorbol myristate acetate in platelets is not due to inhibition of arachidonic acid uptake or incorporation into phospholipids. *Biochim. Biophys. Acta* **1091**, 115-119.

Barna, B.P., Doedhar, S., Gautam, S., Yen-Lieberman, B., and Roberts, D. (1984) Macrophage activation and generation of tumoricidal activity by liposome associated human C-reactive protein. *Cancer Res.* **44**, 305-310.

Barnum, S., Narkates, A.J., Suddath, F.L., and Volanakis, J.E. (1982) Comparative studies of C-reactive protein (CRP) and HOPC 8. *Ann. N. Y. Acad. Sci.* **389**, 431-434.

Baron, B.M., and Limbird, L.E. (1988) Human platelet phospholipase A_2 activity is responsive *in vitro* to pH and Ca^{2+} variations which parallel those occurring after platelet activation *in vivo*. *Biochim. Biophys. Acta* **971**, 103-111.

Baumgarter, H.R., and Born, G.V.R. (1968) Effects of 5-hydroxytryptamine on platelet aggregation. *Nature* **218**, 137-141.

Beardsley, D.S. (1990) Platelet membrane glycoproteins: role in primary hemostasis and component antigens. *Yale J. Biol. Med.* **63**, 469-475.

Becker, R.C. (1991) Seminars in thrombosis and Vascular biology. 3) Platelet activity in Cardiovascular disease. *Cardiology* **79**, 49-63.

Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. (1979) Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. USA* **76**, 3238-3241.

Benveniste, J., Henson, P.M., and Cochrane, C.G. (1972) Leukocyte-dependent histamine release from rabbit platelets. The role for IgE, basophils and a platelet-activating factor. *J. Exp. Med.* **136**, 1356-1376.

Benveniste, J., Tence, M., Varenne, P., Bidault, J., Boullet, C., and Polonsky, J. (1979) Semisynthese et structure proposee du facteur activant les plaquettes (P.A.F.): PAF-acether, un alkyl ether analogue des la lyso-phophatidyl-choline. *C.R. Acad. Sci. Paris* **289**, 1037-1040.

Berridge, M.J. (1984) Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**, 345-360.

Berridge, M.J (1987) Inositol triphosphate and diacylglycerol: two interacting second messengers. *Ann. Rev. Biochem.* **56**, 159-193.

Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P., and Irvine, R.F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.* **212**, 473-482.

Berridge, M.J., and Irvine, R.F. (1984) Inositol triphosphate, A novel second messenger in cellular signal transduction. *Nature* **312**, 315-321.

Best, L.C., Martin, T.J., Russell, R.G.G. and Preston, F.E. (1977) Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets. *Nature* **267**, 850-851.

Besterman, J.M., Duronio, V., and Cuatrecasas, P. (1986) Rapid formation of diacylglycerol from phosphatidylcholine: A pathway for generation of a second messenger. *Proc. Natl. Acad. Sci. USA* **83**, 6785-6789.

Bevers, E.M., Comfurius, P., and Zwaal, R.F.A. (1983) Changes in membrane phospholipid distribution during platelet activation. *Biochim. Biophys. Acta* **736**, 57-66.

Bevers, E.M., Verhallen, P.F.J., Visser, A.J.W.G., Comfurius, P., and Zwaal, R.F.A. (1990) Bidirectional transbilayer lipid movement in human platelets as visualized by fluorescent membrane probe 1-[4-(trimethylammmonio)phenyl]-6-phenyl-1,3,5-hexatriene. *Biochemistry* **29**, 5132-5137.

Billah, M.M., and Anthes, J.C. (1990) The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem J.* **269**, 281-291.

Billah, M.M., Eckel, S., Mullmann, T.J., Egan, R.W., and Siegel, M.I. (1989a) Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. Involvement of phosphatidate phosphohydrolase in signal transduction. *J. Biol. Chem.* **264**, 17069-17077.

Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1979) Phosphatidylinositol-specific phospholipase-C of platelets: association with 1,2-diacylglycerol kinase and inhibition of cyclic-AMP. *Biochem. Biophys. Res. Comm.* **90**, 92-98.

Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1980) Phospholipase A_2 and phospholipase C activities of platelets. Differential substrate specificity, Ca^{2+} requirement, pH dependence, and cellular localization. *J. Biol. Chem.* **255**, 10227-10231.

Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1981) Phospholipase A_2 activity specific for phosphatidic acid. A possible mechanism for the production of arachidonic acid in platelets. *J. Biol. Chem.* **256**, 5399-5403.

Billah, M.M., Pai, J.-K., Mullman, T.J., Egan, R.W., and Siegel, M.I. (1989b) Regulation of phospholipase D in HL-60 granulocytes. *J. Biol. Chem.* **264**, 9069-9076.

Blank, M.L., Snyder, F., Byers, L.W., Brooks, B., and Muirhead, E.E. (1979) Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. *Biochem. Biophys. Res. Comm.* **90**, 1194-1200.

Bleasdale, J.E., Thakur, N.R., Gremban, R.S., Bundy, G.L., Fitzpatrick, F.A., Smith, R.J., and Bunting, S. (1990) Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J. Pharmacol. Exp. Therapeut.* **255**, 756-768.

Bligh, E.G., and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. (1959) *Can. J. Biochem. Physiol.* **37**, 911-918.

Bocckino, S.P., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987) Phosphatidate accumulation in hormone-treated hepatocytes via a phospholipase D mechanism. *J. Biol. Chem.* **262**, 15309-15315.

Born, G.V.R. (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* **194**, 927-929.

Born, G.V.R. and Hume, M. (1967) Effects of the number and sizes of platelet aggregates on the optical density of plasma. *Nature* **215**, 1027-1029.

Bout, D., Joseph, M., Pontet, M., Vorng, H., Deslee, D., and Capron, A. (1986) Rat resistance to schistomaisis: platelet-mediated cytotoxicity induced to C-reactive protein. *Science* **231**, 153-156.

Braquet, P., Touqui, L., Shen, T.Y. and Vargaftig, B.B. (1987) Perspectives in platelet-activating factor research. *Pharmacol. Rev.* **39**, 97-145.

Brass, L.F., Shaller, C.C., and Belmonte, E.J. (1987) Evidence that the activation of phospholipase C-mediated by platelet thromboxane receptors involves a guanine nucleotide binding protein-dependent mechanism distinct from that of thrombin. Inositol-1,4,5-triphosphate induced granule secretion in platelets. *J. Clin. Invest.* **79**, 1269-1275.

Broekman, M.J. (1986) Stimulated platelets release equivalent amounts of arachidonate from phosphatidylcholine, phosphatidylethanolamine, and inositides. *J. Lipid Res.* **27**, 884-891.

Buchta, R., Fridkin, M., Pontet, M., Contessi, E., Scaggiante, B., and Romeo, D. (1987) Modulation of human neutrophil function by C-reactive protein. *Eur. J. Biochem.* **163**, 141-146.

Buchta, R., Gennaro, R., Pontet, M., Fridkin, M., and Romeo D. (1988) C-reactive protein decreases protein phosphorylation in stimulated human neutrophils. *FEBS Lett.* 237, 173-177.

Cabana, V.G., Gewurz, H., and Siegel, J.N. (1982) Interaction of very low density lipoproteins (VLDL) with rabbit C-reactive protein. *J. Immunol.* **128**, 2342-2347.

Cargill, D.I., Cohen, D.S., Van Valen, R.G., Klimek, J.J., and Levin, R.P. (1983) Aggregation, release and desensitization induced in platelets from five species by platelet activating factor (PAF). *Thromb. Haemostas.* **49**, 204-207.

Carter, H.R., Wallace, M.A., and Fain, J.N. (1990) Purification and characterization of PLC-β_m, a muscarinic cholinergic related phospholipase C from rabbit brain membrane. *Biochim. Biophys. Acta* **1054**, 119-128.

Carty, D.J., and Gear, A.R.L. (1985) Rapid changes in protein phosphorylation induced by thrombin, ADP, and epinephrine, as studied by quenched flow techniques. *Thromb. Haemostas.* **54**, 14.

Cathcart, E.S., Comerford, F.R., and Cohen, A.S. (1965) Immunologic studies on a protein extracted from human secondary amyloid. *New Engl. J. Med.* **273**, 143-146.

Chalifa, V., Mohn, H., and Liscovitch, M. (1990) A neutral phospholipase D activity from rat brain synaptic plasma membranes. Identification and partial characterization. *J. Biol. Chem.* **265**, 17512-17519.

Charo, I.F., Feinman, R.D., and Detwiler, T.C. (1977) Interrelations of platelet aggregation and secretion. *J. Clin. Invest.* **60**, 866-873.

Choudhury, S., Macko, S., and Rahimtula, A.D. (1987) Inhibition of rat platelet aggregation by a Purdhoe Bay crude oil and its aliphatic, aromatic, and Heterocyclic fractions. *Toxicol. Appl. Pharmacol.* **90**, 347-356.

Clark, M.A., Littlejohn, D., Conway, T.M., Mong, S., Steiner, S., and Crooke, S.T. (1986a) Leukotriene D_4 treatment of bovine aortic endothelial cells and murine smooth muscle cells in culture results in an increase on phospholipase A_2 activity. *J. Biol. Chem.* **261**, 10713-10718.

Clark, M.A., Shorr, R.G.L, and Bomalaski, J.S. (1986b) Antibodies prepared to *Bacillus cereus* phospholipase C crossreact with a phosphatidylcholine preferring phospholipase C in mammalian cells. *Biochem. Biophys. Res. Comm.* **140**, 114-119.

Claus, D.R., Siegel, J., Petras, K., Osmand, A.P., and Gerwurz, H. (1977) Interactions of C-reactive protein with the first component of human complement. *J. Immunol.* **119**, 187-192.

Cockcroft, S. (1984) Ca^{2+}-dependent conversion of phosphatidylinositol to phosphatidate in neutrophils stimulated with fMet-Leu-Phe or ionophore A23187. *Biochim. Biophys. Acta* **795**, 37-46.

Coe, J.E. (1977) A sex-linked protein of syrian hamster: definition of female protein and regulation by testosterone. *Proc. Natl. Acad. Sci USA* **74**, 730-733.

Coe, J.E. (1982) Female protein of the syrian hamster: a homolog of C-reactive protein. *Ann. N.Y. Acad. Sci.* **389**, 299-305.

Coe, J.E. (1983) Homologs of CRP: A diverse family of proteins with similar structure *In* "Contemporary Topics in Molecular Immunology" Vol. **9**, (Inman, F.P. and Kindt, T.J., eds.) Plenum Publishing Corporation, p211-238.

Colman, R.W. (1990) Aggregin: a platelet ADP receptor that mediates activation. *FASEB J.* **4**, 1425-1435.

Colman, R.W. (1991) Receptors that activate platelets. *Soc. Exp. Biol. Med.* (**Platelet agonists receptors**), 242-248.

Costello, M., Fiedel, B.A., and Gewurz, H. (1979) Inhibition of platelet aggregation by native and desialised alpha-1 acid glycoprotein. *Nature* **281**, 677-678.

Croft, S.M., Mortensen, R.F., and Gewurz, H. (1976) Binding of C-reactive protein to antigen-induced but not mitogen-induced T lymphoblasts. *Science* **193**, 685-687.

Croset, M. and Lagarde, M. (1983) Stereospecific inhibition of PGH_2-induced aggregation by lipoxygenase products of icosaenoic acid. *Biochem. Biophys. Res. Comm.* **112**, 878-883.

Cutler, L., Rodan, G., and Feinstein, M.B. (1978). Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. *Biochim. Biophys. Acta* **542**, 357-371.

Dabrowska, R., and Hartshorne, D.J. (1978) Calcium and modulator-dependent myosin light chain kinase from non-muscle cells. *Biochem. Biophys. Res. Comm.* **85**, 1352-1359.

Daniel, J.L., Molish, I.R., Rigmaiden, M. and Stewart, G. (1984) Evidence for a role of myosin phosphorylation in ADP-induced platelet shape change. *J. Biol. Chem.* **259**, 9826-9831.

Das, S., and Rand, R.P. (1984) Diacylglyerol causes major structural transitions in phospholipid bilayer membranes. *Biochem. Biophys. Res. Comm.* **124**, 491-496.

De Beer, F.C., Baltz, M.L., Munn, E.A., Feinstein, A., Taylor, J., Bruton, C., Clamp, J.R., and Pepys, M.B. (1982a) Isolation and characterization of C-reactive protein and serum amyloid P component in the rat. *Immunology* **45**, 55-70.

De Beer, F.C., Soutar, A.K., Baltz, M.L., Trayner, I.M., Feinstein, A., and Pepys M.B. (1982b) Low density lipoprotein and very low density lipoprotein are selectively bound by aggregated C-reactive protein. *J. Exp. Med.* **156**, 230-242.

De Clerck, F. (1990) The role of serotonin in thrombogenesis. *Clin. Physiol. Biochem.* **8**, 40-49.

Demopoulos, C.A., Pinckard, R.N., and Hanahan, D.J. (1979) Platelet activating factor. Evidence for 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J. Biol. Chem.* **254**, 9355-9358.

Di Camelli, R., Potempa, L.A., Siegel, J., Suyehira, L., Petras, K., and Gewurz, H. (1980) Binding reactivity of C-reactive protein for polycations. *J. Immunol.* **125**, 1933-1938.

Dobrinich, R., and Spagnuolo, P.J. (1991) Binding of C-reactive protein on human neutrophils. Inhibition of respiratory burst activity. *Arthrit. Rheum.* **34**, 1031-1038.

Domino, S.E., Bocckino, S.B., and Garbers, D.L. (1989) Activation of phospholipase C by the fucose-sulfate glycoconjugate that induces an acrosome reaction in spermatozoa. *J. Biol. Chem.* **264**, 9412-9419.

Dougherty, T.J., Gewurz, H., and Siegel, J.N. (1991) Preferential binding and aggregation of rabbit C-reactive protein with arginine-rich proteins. *Mol. Immunol.* **28**, 1113-1120.

Dowton, S.B., Woods, D.E., Mantzouranis, E.C., and Colten, H.R. (1980) Syrian hamster female protein: Analysis of female protein primary structure and gene expression. *Science* **228**, 1206-1208.

Du Clos, T.W. (1989) C-reactive protein reacts with the U1 small nuclear ribonucleoprotein. *J. Immunol.* **143**, 2553-2559.

Du Clos, T.W., Marnell, L., Zlock, L.R., and Burlingame, R.W. (1991) Analysis of the binding of C-reactive protein to chromatin subunits. *J. Immunol.* **146**, 1220-1225.

Du Clos, T. W., Mold, C., Paterson, P.Y., Alroy, J., and Gewurz. H. (1981) Localization of C-reactive protein in inflammatory lesions of experimental allergic encephalomyelitis. *Clin. Exp. Immunol.* **43**, 565-573.

Du Clos, T. W., Mold, C., and Stump, R.F. 1990. Identification of a polypeptide sequence that mediates nuclear localization of the acute phase protein C-reactive protein. *J. Immunol.* **145**, 3869-3875.

Duronio, V., Nip, L. and Pelech, S.L. (1989) Interleukin 3 stimulates phosphatidylcholine turnover in a mast/megakaryocyte cell line. *Biochem. Biophys. Res. Comm.* **164**, 804-808.

Ebbe, S. (1976) Biology of megakaryocytes. *Prog. Hemost. Thromb.* **3**, 211-229.

Edgar, A.D., and Freysz, L. (1982) Phospholipase D activity in the rat brain cytosol. Occurrence of phospholipase C activity with phosphatidylcholine. *Biochim. Biophys. Acta* **711**, 224-228.

Exton, J.H. (1990) Signalling through phosphatidylcholine breakdown. *J.Biol. Chem.* **265**, 1-4.

Farr, R.S., Wardlow, M.L., Cox, C.P., Meng, K.E., and Greene, D.E. (1983) Human serum acid-labile factor is an acylhydrolase that inactivates platelet activating factor. *Fed. Proc.* **42**, 3120-3122.

Fernandez-Moran, H., Marchalonis, J.J., and Edelman, G.M. (1968) Electron microscopy of a hemagglutinin from *Limulis polyphemus*. *J. Mol. Biol.* **32**, 467-469.

Fiedel, B.A. (1984) Interaction of acute phase reactants α_1-acid glycoprotein, C-reactive protein and serum amyloid P-component with platelets and the coagulation system. *Marker Proteins In Inflammation* **2**, 99-124.

Fiedel, B.A. (1985) Platelet agonist synergism by the acute phase reactant C-reactive protein. *Blood* **65**, 264-269.

Fiedel, B.A. (1988) Platelet response to aggregated C-reactive protein: fibrinogen-dependent and independent signals. *Immunology* **63**, 169-170.

Fiedel, B.A., and Gewurz, H. (1976a) Effects of C-reactive protein on platelet function. I. Inhibition of platelet aggregation and release reactions. *J. Immunol.* **116**, 1289-1294.

Fiedel, B.A., and Gewurz, H. (1976b) Effects of C-reactive protein on platelet function. II. Inhibition by CRP of platelet reactivities stimulated by poly-L-lysine, ADP, epinephrine, and collagen. *J. Immunol.* **117**, 1073-1078.

Fiedel, B.A., and Gewurz, H. (1986) Cleaved forms of C-reactive protein are associated with platelet inhibition. *J. Immunol.* **136**, 2551-2555.

Fiedel, B.A., Osmand, A.P., and Gewurz, H. (1976) Inhibition of platelet aggregation by a myeloma protein with anti-phosphorylcholine specificity. *Nature* **263**, 687-689.

Fiedel, B.A., Potempa, L.A., Frenzke, M., Simpson, R.M., and Gewurz, H. (1982a) Platelet inhibitory effects of CRP preparations are due to a co-isolating low molecular weight factor. *Immunology* **45**, 15-22.

Fiedel, B.A., Siegel, J.N., Gewurz, H., Simpson, R.M., and Izzi, J.M. (1982b) Comparison of the enzymatic sensitivities of the platelet receptor for human C-reactive protein and its functional relationship to the platelet IgG Fc receptor. *Clin. Exp. Immunol.* **50**, 215-222.

Fiedel, B.A., Simpson, R.M., and Gewurz, H. (1977) Effects of C-reactive protein on platelet function. III. The role of cAMP, contractile elements, and prostaglandin metabolism in CRP-induced inhibition of platelet aggregation and secretion. *J. Immunol.* **119**, 877-882.

Fiedel, B.A., Simpson, R.M., and Gewurz, H. (1982c) Effects of C-reactive protein (CRP) on platelet function. *Ann. N.Y. Acad. Sci.* **389**, 263-273.

Fiedel, B.A., Simpson, R.M., and Gewurz, H. (1982d) Activation of platelets by modified C-reactive protein. *Immunology* **45**, 439-448.

Filep, J.G., Herman, F., Kelemen, E., and Foldes-Filep, E. (1991) C-reactive protein inhibits binding of platelet-activating factor to human platelets. *Thromb. Res.* **61**, 411-421.

Fisher, G.J., Bakshian, S. and Baldassare, J.J. (1985) Activation of human platelets by ADP causes a rapid use of cytosolic free calcium without hydrolysis of phosphatidylinositol-4,5-bisphosphate. *Biochem. Biophys. Res. Comm.* **129**, 958-964.

Fletcher, T.C., White, A., Youngson, A. Pusztai, A., and Baldo, B.A. (1981) Characterization of C-reactive protein from the eggs of the marine teleost *Cyclopterus lumpus* L. *Biochim. Biophys. Acta* **671**, 44-49.

Floyd-Smith, G., Whitehead, A.S., Colten, H.R., and Francke, U. (1986) The human C-reactive protein (CRP) gene and serum amyloid gene (APCS) are located on the proximal long arm of chromosome 1. *Immunogenetics* **24**, 171-176.

Friesen, L.L., and Gerrard, J.M. The effects of 1-oleoyl-2-acetyl-glycerol. (1985) *Am. J. Pathol.* **121**, 79-87.

Frujmovic, M.M., and Milton, J.G. (1982) Human platelet size, shape, and related functions in health and disease. *Physiol. Rev.* **62**, 185-261.

Ganapathi, M.K., May, L.T., Shultz, D., Brabenec, A., Weinstein, J., Sehgal, P.B., and Kushner, I. (1988) Role of interleukin-6 in regulating synthesis of C-reactive protein and serum amyloid A in human hepatoma cell lines. *Biochem. Biophys. Res. Comm.* **157**, 271-277.

Ganrot, P.O., and Kindmark, C.O. (1969) C-reactive protein--a phagocytosis-promoting factor. *Scand. J. Clin. Lab. Invest.* **24**, 215-219.

Gerrard, J.M., Israels, S.J., and Friesen, L.L. (1985) Platelet protein phosphorylation and platelet ultrastructure. *Nouv. Rev. Fr. Hematol.* **27**, 267-273.

Gerrard, J.M., White, J.G., and Peterson, D.A. (1978) The platelet dense tubular system: its relationship to prostaglandin synthesis and calcium flux. *Thromb. Haemostas.* **40**, 224-231.

Gewurz, H., Mold, C., Siegel, J., and Fiedel, B. (1982) C-reactive protein and the acute phase response. *Adv. Intern. Med.* **27**, 345-372.

Goldman, N.D., and Liu, T.-Y (1987) Biosynthesis of human C-reactive protein in cultured hepatoma cells is induced by monocyte factor(s) other than interleukin-1. *J. Biol. Chem.* **262**, 2363-2368.

Goldman, N., Liu, T., and Lei, K.J. (1987) Structural analysis of the locus containing the human C-reactive protein gene and its related pseudogene. *J. Biol. Chem.* **262**, 7001-7005.

Gotschlich, E.C., and Edelman, G.M. (1965) C-reactive protein. A molecule composed of subunits. *Proc. Natl. Acad. Sci. USA* **54**, 558-566.

Greco, N.J., and Jamieson, G.A. (1991) High and moderate affinity pathways for α thrombin-induced platelet activation. *Proc. Soc. Exp. Biol. Med.* **198**, 792-799.

Halushka, P.V., Mais, D.E., and Saussy, D.L. (1987) Platelet and vascular smooth muscle thromboxane A_2/prostaglandin H_2 receptors. *Fed. Proc.* **46**, 149-153.

Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. USA* **72**, 2994-2998.

Hamberg, M., Svensson, J., Wakabayashi, T. and Samuelsson, B. (1974) Isolation and structure of two prostaglandins endoperoxides that cause platelet aggregation. *Proc. Natl. Acad. Sci. USA* **71**, 345-349.

Hanahan, D.J., and Chaikoff, I.L. (1947) The phosphorus-containing lipides of the carrot. *J. Biol. Chem.* **168**, 233-240.

Harfenist, E.J., Guccione, M.A., Packham, M.A., and Mustard, J.F. (1982) The use of the synthetic peptide, Gly-Pro-Arg-Pro, in the preparation of thrombin-degranulated rabbit platelets. *Blood* **59**, 952-955.

Haslam, R.J., Davidson, M.M.L., Davies, T., Lynham, J.A., and McClenaghan, M.D. (1978a) Regulation of blood platelet function by cyclic nucleotides. *Adv. Cyclic Nucleotide Res.* **9**, 533-552.

Haslam, R.J., Davidson, M.M.L. and Desjardins, J.V. (1978b) Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. *Biochem. J.* **176**, 83-95.

Hathaway, D.R., and Adelstein, R.S. (1979) Human platelet myosin light chain kinase requires the calcium binding protein calmodulin for activity. *Proc. Natl. Acad. Sci. USA* **76**, 1643-1657.

Hawiger, J., Steer, M.L., and Salzman, E.W. (1987) Intracellular regulatory processes in platelets. *In* "Hemostasis and thrombosis: Basic principles and clinical practice", 2nd edn. (Colman, R.W., Hirsh, J., Marder, V.J., Salzman, E.W. eds.) J.W. Lippincott, Philadelphia, p710-725.

Hayakawa, M., Kudo, I., Tomita, M., and Inoue, K. (1988) Purification and characterization of membrane-bound phospholipase A_2 from rat platelets. *J. Biochem.* **103**, 263-266.

Henson, P.M., and Ginsberg, M.H. (1981) Immunological reactions of platelets. *In* "Platelets in biology and pathology II". (Gordon, J.L., ed.) Oxford: Elsevier/North Holland, p107-145.

Higginbotham, J.D., Heidelberger, M., and Gotschlich, E.C. (1970) Degradation of a pneumococcal type-specific polysaccharide with exposure of group-specificity. *Proc. Natl. Acad. Sci USA* **67**, 138-142.

Hokama, Y., Coleman, M.K., and Riley, R.F. (1962) *In vitro* effects of C-reactive protein on phagocytosis. *J. Bacteriol.* **83**, 1017-1024.

Hokama, Y., Garcia, D.A., Bonilla, M.R., Lam, M.P., and Shimizu, R.M. (1984) Inhibition of platelet aggregation by 1-O-alkyl-2-O-acetyl-SN-glycero-3-phosphocholine (PAF) and related analogs. *Fed. Proc.* **43**, abs# 461a.

Holme, S. (1986) *In vitro* platelet responses: shape change. *In* "Platelet responses and metabolism". Vol. 1 (Holmsen, H., ed) CRC Press, Boca Raton, p81-96.

Holmsen, H. (1987) Platelet secretion. *In* "Haemostasis and thrombosis". 2nd edn. (Colman, R.W., Hirch, J., Marder, V.J., Salzman, E.W., eds.) Lippincott, Philadelphia, p606-617.

Holmsen, H. (1989) Physiological functions of platelets. *Ann. Med.* **21**, 23-30.

Holmsen, H. and Dangelmaier, C.E. (1989) Measurement of secretion of serotonin. *Meth. Enzymol.* **169**, 205-210.

Horigome, K., Hayakawa, M., Inoue, K., and Nojima, S. (1987) Selective release of phospholipase A_2 and lysophosphatidylserine-specific lysophospholipase from rat platelets. *J. Biochem.* **101**, 53-61.

Hornung, M. (1972) Growth inhibition of human melanoma cells by C-reactive protein (CRP) activated lymphocytes. *Proc. Soc. Exp. Biol. Med.* **139**, 1166-1169.

Hrbolich, J.K., Culty, M., and Haslam, R.J. (1987) Activation of phospholipase C associated with isolated rabbit platelet membranes by guanosine 5'-[Δ-thio] triphosphate and by thrombin in the presence of GTP. *Biochem J.* **243**, 457-465.

Huang, C., and Cabot, M.C. (1990) Phorbol diesters stimulate the accumulation of phosphatidate, phosphatidylethanol and diacylglycerol in three cell types. Evidence for the indirect formation of phosphatidylcholine-derived diacylglycerol by a phospholipase D pathway and direct formation of diacylglycerol by a phospholipase C pathway. *J. Biol. Chem.* **265**, 14858-14863.

Huang, E.M., and Detwiler, T.C. (1986) Stimulus-response coupling mechanisms. *In* "Biochemistry of platelets" (Phillips, D.R., and Shuman, M.A., eds.) Academic Press Inc., Orlando, p1-68.

Huang, R., Kucera, G.L., and Rittenhouse, S.E. (1991) Elevated cytosolic Ca^{2+} activates phospholipase D in human platelets. *J. Biol. Chem.* **266**, 1652-1655.

Hwang, S.B. (1990) Specific receptors of platelet activating factor, receptor heterogeneity, and signal transduction mechanisms. *J. Lipid Mediators* **2**, 123-158.

Hwang, S.B., Lam, M.H., and Pong, S.S. (1986) Ionic and GTP regulation of binding of platelet-activating factor to receptors and platelet-activating factor-induced activation of GTPase in rabbit platelet membranes. *J. Biol. Chem.* **261**, 532-537.

Hwang, S.B., Lee, C.S.C., Cheah, M.J., and Shen, T.Y. (1983) Specific receptor sites for 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet activating factor) on rabbit platelet and quinea pig smooth muscle membranes. *Biochemistry* **22**, 4756-4763.

Ikuta, T., Okubo, H., Ishibashi, H., Okumura, Y., and Hayashida, K. (1986) Human lymphocytes synthesize C-reactive protein. *Inflammation* **10**, 223-232.

Imai, A., Ishizuka, Y., Kawai, K., and Nozawa, Y. (1982) Evidence for coupling of phosphatidic acid formation and calcium influx in thrombin-stimulated platelets. *Biochem. Biophys. Res. Comm.* **108**, 752-759.

Inarrea, P., Gomez-Cambronero, J., Neito, M., and Sanchez-Crespo, M. (1984) Characteristics of the binding of Platelet-activating factor to platelets of different animal species. *Eur. J. Pharmacol.* **105**, 309-315.

Irving, H.R., and Exton, J.H. (1987) Phosphatidylcholine breakdown in rat liver plasma membrane. Roles of guanine nucleotides and P_2-purinergic agonists. *J. Biol. Chem.* **262**, 3440-3443.

Irvine, R.F. (1982) How is the level of free arachidonic acid controlled in mammalian cells. *Biochem. J.* **204**, 3-16.

Isreals, S.J., Robinson, R., Docherty, J.C., and Gerrard, J.M. (1985) Activation of permeabilized platelets by inositol-1,4,5-triphosphate. *Thromb Res.* **40**, 499-509.

Jamaluddin, M.P., and Krishnan, L.K. (1987) A spectrophotometric method for following initial rate kinetics of blood platelet aggregation. *J. Biochem. Biophys. Meth.* **14**, 191-200.

Jennings, H.J., Lugowski, C., and Young, N.M. (1980) Structure of the complex polysaccharide C-substance from *Streptococcus pneumonia* type 1. *Biochemistry* **19**, 4712-4719.

Kabat, E.A., Wu, T.T., and Bilofsky, H. (1976) Attempts to locate residues in complementarity-determining regions of antibody combining sites that make contact with antigen. *Proc. Natl. Acad. Sci. USA* **73**, 617.

Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. *J. Biol. Chem.* **258**, 6701-6704.

Kaplan, M.H., and Volanakis, J.E. (1974) Interaction of C-reactive protein complexes with the complement system. I. Consumption of human complement associated with the reaction of C-reactive protein with pneumococcal C-polysaccharide and with the choline phosphatides, lecithin and sphingomyelin. *J. Immunol.* **112**, 2135-2147.

Kater, L.A., Goetzl, E.J., and Austen, K.F. (1976) Isolation of human eosinophil phospholipase D. *J. Clin. Invest.* **57**, 1173-1180.

Kempka, G., Roos, P.H., and Kolb-Bachofen, V. (1990) A membrane-associated form of C-reactive protein is the galactose-specific particle receptor on rat liver macrophages. *J. Immunol.* **144**, 1004-1009.

Kilpatrick, J.M., Gresham, H.D., Griffin, F.M., and Volanakis, J.E. (1987) Peripheral blood mononuclear leukocytes release a mediator(s) that induces phagocytosis of C-reactive protein-coated cells by polymorphonuclear leukocytes. *J. Leukoc. Biol.* **41**, 150-155.

Kilpatrick, J.M, and Virella, G. (1985) Inhibition of platelet-activating factor by rabbit C-reactive protein. *Clin. Immunol. Immunopathol.* **37**, 276-281.

Kilpatrick, J.M., and Volanakis, J.E. (1985) Opsonic properties of C-reactive protein. Stimulation by phorbol myristate acetate enables human neutrophils to phagocytize C-reactive protein-coated cells. *J. Immunol.* **134**, 3364-3370.

Kilpatrick, J.M, and Volanakis, J.E. (1991) Molecular genetics, structure, and function of C-reactive protein. *Immunol. Res.* **10**, 43-53.

Kindmark, C. O. (1971) Stimulating effect of C-reactive protein on phagocytosis of various species in pathogenic bacteria. *Clin. Exp. Immunol.* **8**, 941-948.

Kiss, Z., and Anderson, W.B. (1989) Phorbol ester stimulates the hydrolysis of phosphatidylethanolamine in leukaemic HL-60, NIH 3T3, and baby hamster kidney cells. *J. Biol. Chem.* **264**, 1483-1487.

Kolb-Bachofen, V. (1991a) A membrane-bound form of the acute phase protein C-reactive protein is the galactose-specific particle receptor on rat liver macrophages. *Pathobiology* **59**, 272-275.

Kolb-Bachofen, V. (1991b). A review on the biological properties of C-reactive protein. *Immunobiology* **183**, 133-145.

Koltai, M., Hosford, D., Guinot, P., Esanu, A., and Braquet, P. (1991a) Platelet activating factor (PAF): A review of its effects, antagonists and possible future clinical implications (Part 1). *Drugs* **42**, 9-29.

Koltai, M., Hosford, D., Guinot, P., Esanu, A., and Braquet, P. (1991b) Platelet activating factor (PAF): A review of its effects, antagonists and possible future clinical implications (Part 2). *Drugs* **42**, 174-204.

Krishnamurthi, S., Patel, Y., and Kakkar, V.V. (1989) Weak inhibition of protein kinase C coupled with various non-specific effects make sphingosine an unsuitable tool in platelet signal transduction studies. *Biochim. Biophys. Acta* **1010**, 258-264.

Kritz, R., Lin, L.-L., Sultzman, L., Ellis, C., Heldin, C.-H., Pawson, T., and Konpf, J. (1990) Phospholipase C isozymes: structure and functional similarities. *In* "Proto-Oncogenes in cell development." Cida Foundation Symposium, John Wiley and Sons, Chichester, p112-127.

Kunicki, T.J., Montgomery, R.R., and Pidard, D. (1983) Structure/function of human glycoprotein 1b (GP1b). *Blood* **62**, 260a.

Kushner, I. (1982) The phenomenon of the acute phase response. *Ann. N.Y. Acad. Sci.* **389**, 39-48.

Kuta, A.E., and Baum, L.L. (1986) C-reactive protein is produced by a small number of normal human peripheral blood lymphocytes. *J. Exp. Med.* **164**, 321-326.

Lagarde, M. (1988) Metabolism of fatty acids by platelets and the functions of various metabolites in mediating platelet function. *Prog. Lipid Res.* **27**, 135-152.

Lanara, E., Vakirtzi-Lemonias, C., Kritikou, L., and Demopoulos, C.A. (1982) Response of mice and mouse platelets to acetyl glyceryl ether phosphorylcholine. *Biochem. Biophys. Res. Comm.* **109**, 1148-1156.

Lapetina, E.G. (1982) Regulation of arachidonic acid production: Role of phospholipase A_2 and C. *Trends Pharmacol. Sci.* **3**, 115-118.

Lapetina, E.G. (1986) Inositol-dependent and independent mechanisms in platelet activation. *In* "Phosphoinositides and receptor mechanisms", (Putney, J.W., ed.) Alan R. Liss Inc., New York, p271-286.

Lapetina, E.G. (1990) The signal transduction induced by thrombin in human platelets. *FEBS Lett.* **268**, 400-404.

Lei, K.J., Liu, T., Zon, G., Soravia, E., Liu, T.Y., and Goldman, N.D. (1985) Genomic DNA sequence for human C-reactive protein. *J. Biol. Chem.* **260**, 13377-13383.

Leon, M.A., and Young, N.M. (1971) Specificity for phosphorylcholine of six murine myeloma proteins reactive with *Pneumococcus* C polysaccharide and ß-lipoprotein. *Biochemistry* **10**, 1424-1429.

Liscovitch, M., and Amsterdam, A. (1989) Gonadotropin-releasing hormone activates phospholipase C in ovarian granulosa cells. Possible role in signal transduction. *J. Biol. Chem.* **264**, 11762-11767.

Litosch, I. and Fain, J.N. (1986) Regulation of phosphoinositide breakdown by guanine nucleotides. *Life Sci.* **39**, 187-194.

Lofstrom, G. (1944) Comparison between the reactions of acute phase serum with pneumococcus C-polysaccharide and with pneumococcus type 27. *Br. J. Exp. Pathol.* **25**, 21-26.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J.Biol.Chem.* **193**, 265-275.

Mackiewicz, S., Wiktorowicz, K., and Mackiewicz, A. (1985) The effect of C-reactive protein on the PHA-induced proliferation of human peripheral blood lymphocytes. *Arch. Immunol. Ther. Exp.* **33**, 603-608.

Magosci, M., Sarkadi, B., Kovacs, T., and Gardos, G. (1989) Thrombin-induced activation of calcium transport pathways and their role in platelet functions. *Biochim. Biophys. Acta* **984**, 88-96.

Marche, P., Limon, I., Blanc, J., and Girard, A. (1990) Platelet phosphatidylcholine turnover in experimental hypertension. *Hypertension* **16**, 190-193.

Martin, T.W. (1988) Formation of diacylglycerol by a phospholipase D-phosphatidate phosphatase pathway specific for phosphatidylcholine in endothelial cells. *Biochim. Biophys. Acta.* **962**, 282-296.

Martin, T.W., and Michaelis, K. (1989) P_2-Purinergic agonists stimulate phosphodiesteric cleavage of phosphatidylcholine in endothelial cells. Evidence for activation of phospholipase D. *J. Biol. Chem.* **264**, 8847-8856.

Martin, T.W., Wysolmerski, R.B., and Lagunoff, D. (1987) Phosphatidylcholine metabolism in endothelial cells: evidence for phospholipase A and a novel Ca^{2+}-independent phospholipase C. *Biochim. Biophys. Acta* **917**, 296-307.

Matozaki, T., and Williams, J.A. (1989) Multiple sources of 1,2-diacylglycerol in isolated rat pancreatic acini stimulated by cholecystokinin. Involvement of phosphatidylinositol bisphosphate and phosphatidylcholine hydrolysis. *J. Biol. Chem.* **264**, 14729-14734.

Matsumoto, M., and Miwa, M. (1985) Platelet-activating factor binding protein in human serum. *Adv. Prostagland. Thrombox. Leukotri. Res.* **15**, 705-706.

Matsuzawa, Y., and Hostetler, K.Y. (1980) Properties of phospholipase C isolated from rat liver lysosomes. *J. Biol. Chem.* **255**, 646-652.

Maudsley, S., Baltz., M.L., Munn, E.A., Buttress, N., Herbert, J., Feinstein, A., and Pepys, M.B. (1987a) Isolation and characterization of goat C-reactive protein. *Biochim. Biophys. Acta.* **924**, 75-80.

Maudsley, S. Rowe, L.F., De Beer, F.C., Munn, E.A., Buttress, N., Herbert, J., Feinstein, A., and Pepys, M. (1987b) Identification and isolation of two pentraxins from bovine serum. *Clin. Exp. Immunol.* **67**, 662-673.

Mazur, E.M. (1987) Megakaryocytopoiesis and platelet production: a review. *Exp. Hematol.* **15**, 340-350.

McCarty, M. (1947) The occurrence during acute infections of a protein not normally present in blood. IV. Crystallization of the C-reactive protein. *J. Exp. Med.* **85**, 491-498.

McEver, R.P. (1990) The clinical significance of platelet membrane glycoproteins. *Haematol. Oncol. Clin. N. A.* **4**, 87-105.

McNamara, P.J., Brouwer, K.R., and Gillespie, M.N. (1986) Autocoid binding to serum proteins. Interaction of platelet activating factor (PAF) with human serum alpha-1-acid glycoprotein (AAG). *Biochem. Pharmacol.* **35**, 621-624.

Meldrum, E., Parker, P.J. and Carozzi, A. (1991) The PtdIns-PLC superfamily and signal transduction. *Biochim. Biophys. Acta* **1092**, 49-71.

Merritt, J.E., and Hallam, T.J. (1988) Platelets and parotid acinar cells have different mechanisms for agonist-stimulated divalent cation entry. *J. Biol. Chem.* **263**, 6161-6164.

Michell, R.H. (1975) Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta* **415**, 81-147.

Mills, D.C.B. (1982) Cyclic nucleotides in blood platelets. *In* "Handbook of experimental pharmacology" Vol. **58**, (Kebabian, J.W., and Nathanson, J.A., eds), Springer-Verlag, Berlin and New York, p723-761.

Mills, D.C.B., Figures, W.R., Scearce, L.M., Stewart, G.J., Colman, R.F., and Colman, R.W. (1985) Two mechanisms for inhibition of ADP-induced platelet shape change by 5'-*p*-florosulfonylbenzoyl adenosine. Conversion to adenosine, and covalent modification at an ADP binding site distinct from that which inhibits adenylate cyclase. *J. Biol. Chem.* **260**, 8078-8083.

Miyagawa, K., Kiyono, S., and Inoue, K. (1988) Modulation of stimulus-dependent human platelet activation by C-reactive protein modified with active oxygen species. *J. Immunol.* **141**, 570-574.

Mookerjea, S. (1978) Mechanism of heparin and serum lipoprotein interactions: Effects of calcium, phosphorylcholine, and a serum factor. *Can. J. Biochem.* **56**, 746-752.

Morrison, W.J., and Shukla, S.D. (1988) Desensitization of receptor-coupled activation of phosphoinositide-specific phospholipase C in platelets: evidence for distinct mechanisms for platelet-activating factor and thrombin. *Mol. Pharmacol.* **33**, 58-63.

Mortenson, R.F. (1982) Inhibition of the polyclonal antibody plaque-forming cell: response of human B lymphocytes by C-reactive protein (CRP) and CRP complexes. *Cell. Immunol.* **66**, 99-110.

Mortensen, R.F., Braun, K., and Gewurz, H. (1977) Effects of C-reactive protein on lymphocyte functions. III. Inhibition of antigen-induced lymphocyte stimulation and lymphokine production. *Cell Immunol.* **28**, 59-68.

Mortensen, R. F., Osmand, A. P., and Gewurz, H. (1975) Effects of C-reactive protein on the lymphoid system. I. Binding to thymus-dependent lymphocytes and alterations of their function. *J. Exp. Med.* **141**, 821-839.

Mortensen, R.F., Osmand, A.P., Lint, T.F. and Gewurz, H. (1976) Interaction of C-reactive protein with lymphocytes and monocytes: Complement-dependent adherence and phagocytosis. *J. Immunol.* **117**, 774-781.

Moshage, H.J., Roelofs, H.M.J., van Pelt, J.F, Hazenberg, B.P.C., Van Leeuwen, Limburg, P.C., Aarden, L.A., and Yap, S.H. (1988) The effect of Interleukin-1, Interleukin-6 and its interrelationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. *Biochem. Biophys. Res. Comm.* **155**, 112-117.

Muller, H., and Fehr, J. (1986) Binding of C-reactive protein to human polymorphonuclear leukoctes: evidence for association of binding sites with Fc receptors. *J. Immunol.* **136**, 2202-2207.

Murphy, T.M., Baum, L.L, and Beaman K.D. (1991) Extrahepatic transcription of human C-reactive protein. *J. Exp. Med.* **173**, 495-498.

Nagpurkar, A., and Mookerjea, S. (1981) A novel phosphorylcholine-binding protein from rat serum and its effect on heparin-lipoprotein complex formation in the presence of calcium. *J. Biol. Chem.* **256**, 7440-7448.

Nagpurkar, A., Hunt, D., Yang, C., and Mookerjea, S. (1992) Degradation of rat C-reactive protein by macrophages. (Submitted to *Biochem. J.*)

Nagpurkar, A., Saxena, U., and Mookerjea, S. (1983) Interaction of rat serum phosphorylcholine-binding protein with phospholipid-containing liposomes. *J. Biol. Chem.* **258**, 10518-10523.

Nakano, T., Terawaki, A., and Arita, H. (1987) Signal transduction in collagen-stimulated rat platelets is composed of three stages. *J. Biochem.* **101**, 1169-1180.

Nakashima, S., Suganuma, A., Matsui, A., and Nozawa, Y. (1991) Thrombin induces a biphasic 1,2-diacylglycerol production in human platelets. *Biochem. J.* **275**, 355-361.

Nakashima, S., Tohmatsu, T., Hattori, H., Suganuma, A., and Nozawa, Y. (1987) Guanine nucleotides stimulate arachidonic acid release by phospholipase A_2 in saponin-permeabilized human platelets. *J. Biochem.* **101**, 1055-1058.

Nazih, H., Devred, D., Martin-Nizard, F., Fruchart, J.C., and Delbart, C. (1990) Phosphatidylcholine breakdown in HDL_3 stimulated platelets. *Thromb. Res.* **59**, 913-920.

Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., and Lefkowith, J.B. (1986) Arachidonic acid metabolism. *Ann. Rev. Biochem.* **55**, 69-102.

Nishakawa, M., Tanaka, T., and Hidaka, H. (1980) Ca^{2+}-calmodulin dependent phosphorylation and platelet secretion. *Nature* **287**, 863-865.

Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour production. *Nature* **308**, 693-698.

Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* **233**, 305-312.

Nishizuka, Y. (1989a) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665.

Nishizuka, Y. (1989b) The family of protein kinase C for signal transduction. *JAMA* **262**, 1826-1833.

Nurden, A.T. (1987) Platelet membrane glycoproteins and their clinical aspects. *In* "Thrombosis and hemostasis", (Verstraete, M., Vermylen, J., Lijnen, R., and Arnout, J. eds.). Leuven University Press, Leuven, p93-125.

Oishi, N., Torikawa, C., Ochiai, H., and Hokama, Y. (1973) Relationship of C-reactive protein in autologous plasma of cancer patients and choline phosphate in lymphocyte responses to phytohemagglutinin. *J. Reticuloendothelial. Soc.* **14**, 242-249.

Oliveira, E.B., Gotschlich, E.C., and Liu, T.Y. (1979) Primary structure of human C-reactive protein. *J. Biol. Chem.* **254**, 489-502.

O'Rourke, F.A., Halenda, S.P., Zavoico, G.B., and Feinstein, M.B. (1985) Inositol-1,4,5-triphosphate release calcium from a Ca^{2+}-transporting membrane vesicle fraction derived from human platelets. *J. Biol. Chem.* **260**, 956-962.

Osmand, A.P., Friedenson, B., Gewurz, H., Painter, R.H., Hofmann, T., and Shelton, E. (1977) Characterization of C-reactive protein and the complement subcomponent C1t as homologous proteins displaying cyclic pentameric symmetry (pentraxins). *Proc. Natl. Acad. Sci. USA* **74**, 739-743.

Osmand, A.P., Mortensen, R.F., Siegel, J., and Gewurz, H. (1975) Interactions of C-reactive protein with the complement system. III. Complement-dependent passive haemolysis initiated by CRP. *J. Exp. Med.* **112**, 1065-1077.

Oster, G.F. and Perelson, A.S. (1987) The physics of cell motility. *J. Cell Sci. Suppl.* **8**, 35-54.

Packham, M.A., and Mustard, J.F. (1984) Normal and abnormal platelet activity. *In* "Blood platelet function and medicinal chemistry", (Lasslo, A., ed.), Elsevier Biomedical, New York, p61-128.

Page, C.P. (1989) Platelets as inflammatory cells. *Immunopharmacol.* **17**, 51-59.

Pai, J.K., Siegel, M.I., Egan, R.W. and Billah, M.M. (1988) Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. *J. Biol. Chem.* **263**, 12472-12477.

Pelech, S.L., Clarest, D.L., Howard, S.L., Paddon, H.B., and Salari, H. (1990) Protein kinase C activation by platelet activating factor is independent of enzyme translocation. *Biochim. Biophys. Acta* **1051**, 100-107.

Pepys, M.B., and Baltz, M.L. (1983) Acute phase proteins with special reference to C-reactive proteins and related proteins (Pentraxins) and serum amyloid A protein. *Adv. Immunol.* **34**, 141-212.

Pepys, M.B, Dash, A.C., Fletcher, T.C., Richardson, N., Munn, E.A., and Feinstein, A. (1978) Analogues in other mammals and in fish of human plasma proteins, C-reactive protein and amyloid P component. *Nature* **273**, 168-170.

Pepys, M.B., De Beer, F.C., Milstein, C.P., March, J.F., Feinstein, A., Buttress, N., Clamp, J.R., Taylor, J., Bruton, C., and Fletcher, T.C. (1982) C-reactive protein and serum amyloid P component in the plaice (*Pleuronectes platessa* L.), a marine teleost, are homologous with their human counterparts. *Biochim. Biophys. Acta* **704**, 123-133.

Pepys, M.B., Rowe, I.F., and Baltz, M.L. (1988) C-reactive protein: binding to lipids and lipoproteins. *Intl. Rev. Exp. Pathol.* **27**, 83-111.

Pollet, R., and Edelhoch, H. (1973) The binding properties of anti-phosphorylcholine mouse myeloma proteins as measured by protein fluorescence. *J. Biol. Chem.* **248**, 5443-5447.

Pollock, W.K., Sage, S.O., and Rink, T.J. (1987) Stimulation of Ca^{2+} from fura-2-loaded platelets activated by thrombin or phorbol myristate acetate. *FEBS Lett.* **210**, 132-136.

Pontet, M., Ayrault-Jarrier, M., Burdin, J., Gelin, M., and Engler, R. (1979) Preparation et form serique de la proteine reactive C de lapin. *Biochimie* **61**, 1293-1299.

Pontet, M., D'Asnieres, M., Gache, D., Escaig, J., and Engler, R. (1981) A new pentraxin (serum amyloid P component) in the rat. Evidence for two quaternary structures and effect of ligands on self association. *Biochim. Biophys. Acta* **671**, 202-210.

Potempa, L.A., Siegel, J., and Gewurz, H. (1981) Binding reactivity of C-reactive protein for polycations. II. Modulatory effects of calcium and phosphocholine. *J. Immunol.* **127**, 1509-1514.

Pritchard, D.G., Volanakis, J.E., Slutsky, G.M., and Greenbalt, C.L. (1985) C-reactive protein binds leishmanial excreted factors. *Proc. Soc. Exp. Biol. Med.* **178**, 500-503.

Puolakkainen, P., Valtonen, V., Paananen, A., and Schroder, T. (1987) C-reactive protein (CRP) and serum phospholipase A_2 in the assessment of severity of acute pancreatitis. *Gut* **28**, 764-771.

Putney, J.W., Weiss, S.J., Van de Walle, C.M., and Haddas, R.A. (1980) Is phosphatidic acid a calcium ionophore under neurohumoral control? *Nature* **284**, 345-347.

Qian, Z., and Drewes, L.R. (1990) A novel mechanism for acetylcholine to generate diacylglycerol in brain. *J. Biol. Chem.* **265**, 3607-3610.

Radley, J.M (1986) Ultrastructural aspects of platelet production. *In* "Megakaryocyte development and function". (Levine, R.F., Williams, N., Levine, J., Evatt, B.L., eds.) Alan R. Liss Inc., New York, p387-398.

Radley, J.M., and Haller, C.J. (1982) The demarcation membrane system of the megakaryocyte: a misnomer? *Blood* **60**, 213-214.

Radley, J.M., and Haller, C.J. (1983) Fate and senescent megakaryocytes in the bone marrow. *Br. J. Haematol.* **53**, 277-287.

Rao, A.K. and Kowalska, M.A. (1987) ADP-induced shape change and mobilization of cytoplasmic ionized calcium are mediated by distinct binding sites on platelets: 5'-p-florosulfonylbenzoyladenosine is a weak platelet agonist. *Blood* **70**, 751-756.

Rassouli, M., Sambasivam, H., Azadia, P., Dell, A., Morris, H.R., Nagpurkar, A., Mookerjea, S., and Murray, R.K. (1992) Derivation of the amino acid sequence of rat C-reactive protein from cDNA cloning with additional studies on the nature of its dimeric component. *J. Biol. Chem.* **267**, 2947-2954.

Remmal, A., Koutouzov, S., and Marche, P. (1988) Enhanced turnover of phosphatidylcholine in platelets of hypertensive rats. Possible involvement of phosphatidylcholine-specific phospholipase C. *Biochim. Biophys. Acta* **960**, 236-244.

Rhee, S.G., Suh, P.-G., Ryu, S.-H, and Lee, S.Y. (1989) Studies of inositol phospholipid-specific phospholipase C. *Science* **244**, 546-550.

Richards, R.L., Gewurz, H., Siegel, J., and Alving, C.R. (1979). Interactions of C-reactive protein and complement with liposomes. II. Influence of membrane composition. *J. Immunol.* **122**, 1185-1189.

Riley, R.F., and Coleman, M.K. (1970) Isolation of C-reactive proteins of man, monkey, rabbit and dog by affinity chromatography on phosphorylated cellulose. *Clin. Chim. Acta.* **30**, 483-496.

Riley, R.F., Hokama, Y., Colver, V., Coleman, M.K., and Dowdy, A.H. (1958) Seroflocculant activity of ethyl choladienate and various alcohols in the Penn test for cancer. *Cancer Res.* **18**, 833-841.

Rink, T.J., and Hallam, T.J. (1984) What turns platelet on? *TIBS* **9**, 215-219.

Rittenhouse, S.E. (1983) Human platelets contain phospholipase C that hydrolyses poly-phosphoinositides. *Proc. Natl. Acad. Sci. USA* **80**, 5417-5420.

Rittenhouse, S.E. (1984) Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclooxygenase products and ATP. *Biochem. J.* **222**, 103-110.

Rittenhouse-Simmons, S. (1979) Production of diglyceride from phosphatidylinositol in activated platelets. *J. Clin. Invest.* **63**, 580-587.

Robey, F.A., Jones, K.D, and Steinberg, A.D. (1985) C-reactive protein mediates the solubilization of nuclear DNA by complement *in vitro*. *J. Exp. Med.* **161**, 1344-1356.

Robey, F.A., Jones, K.D., Tanaka, T. and Liu, T.Y. (1984) Binding of C-reactive protein to chromatin and nucleosome core particles: a possible physiological role of C-reactive protein. *J. Biol. Chem.* **259**, 7311-7316.

Robey, F.A., and Liu, T.Y. (1981) Limulin: a C-reactive protein from *Limulus polyphemus*. *J. Biol. Chem.* **256**, 969-975.

Robey, F.A., Tanaka, T., and Liu, T.Y. (1983) Isolation and characterization of two major serum proteins from the dogfish, *Mustelus canis*, C-reactive protein and amyloid P component. *J. Biol. Chem.* **258**, 3889-3894.

Rosoff, P.M., Savage, N. and Dinarello, C.A. (1988) Interleukin-1 stimulates diacylglycerol production on T-lymphocytes by a novel mechanism. *Cell* **54**, 73-81.

Rouser, G., Kritchevsky, G. and Yamamoto, A. (1976) Column chromatography and associated procedures for separation and determination of phosphatidates and glycolipids. *In* "Lipid chromatographic analysis" 2nd edn. Vol **3**, Dekker, New York, p713-776.

Rowe, I.F., Soutar, A.K., Trayner, I.M., Baltz, M.L., De Beer, F.C., Walker, L., Bowyer,D., Herbert, J., Feinstein, A., and Pepys, M.B. (1984a) Rabbit and rat C-reactive proteins bind apolipoprotein B-containing lipoproteins. *J. Exp. Med.* **159**, 604-616.

Rowe, I.F., Soutar, A.K., Trayner, I.M., Thompson, G.R., and Pepys, M.B. (1984b) Circulating human C-reactive protein binds very low density lipoproteins. *Clin. Exp. Immunol.* **58**, 237-244.

Rubin, R. (1988) Phosphatidylethanol formation in human platelets: evidence for thrombin-induced activation of phospholipase D. (1988) *Biochem. Biophys. Res. Comm.* **156**, 1090-1096.

Sage, S.O. and Rink, T.J. (1987) The kinetics of changes in intracellular calcium concentration on fura-2-loaded human platelets. *J. Biol. Chem.* **262**. 16364-16369.

Saito, M., and Kanfer, J.N. (1975) Phosphatidohydrolase activity in a solubilized preparation of rat brain particulate fraction. *Arch. Biochem. Biophys.* **169**, 318-323.

Sambasivam, H., Rassouli, M., Murray, R.K., Dell, A., Nagpurkar, A., Mookerjea, S., Azadi, P., Morris, H.R., and Dell, A. (1992) Studies on the carbohydrate moiety and on the biosynthesis of rat C-reactive protein (Submitted to *J. Biol. Chem.*)

Sanchez-Crespo, M., Alsonso, F., Inarrea, P., and Egido, J. (1981) Non-platelet-mediated vascular actions of synthetic platelet activating factors. *Agents Actions* **11**, 565-566.

Sane, D.C, Bielawska, A., Greenberg, C.S., Hannun, Y.A. (1989) Cyclic GMP analogues inhibit Gamma thrombin-induced arachidonic acid release in human platelets. *Biochem. Biophys. Res. Comm.* **165**, 708-714.

Saxena, U., Nagpurkar, A., Dolphin, P.J., and Mookerjea, S. (1987) A study of the selective binding of apoprotein B- and E-containing human plasma lipoproteins to immobilized rat serum phosphorylcholine-binding protein. *J. Biol. Chem.* **262**, 3011-3016.

Saxena, U., Nagpurkar, A., and Mookerjea, S. (1985) Contrasting effect of phosphorylcholine-binding protein from rat and rabbit on heparin-lipoprotein interaction: a role of sialic acid. *Can. J. Biochem. Cell Biol.* **63**, 1014-1021.

Saxena, U., Nagpurkar, A., and Mookerjea, S. (1986) Inhibition of the binding of low density lipoproteins to liver membrane receptors by rat serum phosphorylcholine binding protein. *Biochem. Biophys. Res. Comm.* **141**, 151-157.

Schror, K., and Braun, M. (1990) Platelets as a source of vasoactive mediators. *Stroke* **21**, IV-32-IV-35.

Scrutton, M.C. and Knight, D.E. (1987). Permeabilised preprations: their role in studies of signal transduction mechanism. *In* "Platelets in biology and pathology III" (MacIntyre, D.E. and Gordon, J.L., eds.) Elsevier Science Publishers, Amsterdam, p467-492.

Sheikhnejad, R.G., and Srivastava, P.N. (1986) Isolation and properties of phosphatidylcholine-specific phospholipase C from bull seminal plasma. *J. Biol. Chem.* **261**, 7544-7549.

Shepard, E.G., Anderson, R., Beer, S.M., Van Rensbury, C.E.J., and De Beer, F.C. (1988) Neutrophil lysosomal degradation of human CRP: CRP-derived peptides modulate neutrophil function. *Clin. Exp. Immunol.* **73**, 139-145.

Siegel, D.P., Banschbach, J., Alford, D., Ellens, H., Lis, L.J., Quinn, P.J., Yeagle, P.L., and Bentz, J. (1989) Physiological levels of diacylglyerols in phospholipid membranes induce membrane fusion and stabilize inverted phases. *Biochemistry* **28**, 3703-3709.

Siegel, J., Osmand, A.P., Wilson, M.F., and Gewurz, H. (1975) Interactions of C-reactive protein with the complement system. II. CRP-mediated consumption of complement by poly-L-lysine polymers and other polycations. *J. Exp. Med.* **142**, 709-721.

Siegel, J., Rent, R., and Gewurz, H. (1974) Interaction of C-reactive protein with the complement system. I. Protamin-induced consumption of complement in acute phase sera. *J. Exp. Med.* **140**, 631-647.

Siess, W., Cuatrecasas, P., and Lapetina, E.G. (1983) A role for cyclooxygenase products in the formation of phosphatidic acid in stimulated platelets. Differential mechanism of action of thrombin and collagen. *J. Biol. Chem.* **258**, 4683-4686.

Simpson, R.M., Prancan, A., Izzi, J.M., and Fiedel, B.A. (1982) Generation of thromboxane A_2 and aorta-contracting activity from platelets stimulated by modified C-reactive protein. *Immunology* **47**, 193-202.

Skinner, M., and Cohen, A. S. (1988) Amyloid P. Component. *Meth. Enzymol.* **163**, 523-536.

Slivka, S.R., Meier, K.E., and Insel, P.A. (1988) α_1-Adrenergic receptors promote phosphatidylcholine hydrolysis in MDCK-D1 cells. A mechanism for rapid activation of protein kinase C. *J. Biol. Chem.* **263**, 12242-12246.

Snyder, F. (1985) Chemical and biochemical aspects of "platelet activating factor," a novel class of acetylated ether-linked choline-phospholipids. *Med. Res. Rev.* **5**, 107-140.

Soelter, J., and Uhlenbruck, G. (1986) The role of phosphate groups in the interaction of human C-reactive protein with galactan polysaccharides. *Immunology* **58**, 139-144.

Stafforini, D.M., McIntyre, T.M., Carter, M.E., and Prescott, S.M. (1987) Human plasma platelet activating factor acetylhydrolase. Association with lipoprotein particles and role in the degradation of platelet activating factor. *J. Biol. Chem.* **262**, 4215-4222.

Stel, H.V., Sakariassen, K.S., De Groot, P.G., Van Mourik, J.A., and Sixma, J.J. (1985) Von Willebrand factor in the vessel wall mediates platelet adherence. *Blood* **65**, 85-90.

Stenberg, P.E., and Bainton, D.F. (1987) Storage organelles in platelets and megakaryocytes. *In* "Biochemistry of platelets", (Phillips, D.R., and Shuman, M.A., eds.) Academic Press Inc., Orlando, p257-295.

Stenberg, P.E., and Levin, J. (1989) Mechanisms of platelet production. *Blood Cells* **15**, 23-47.

Sugatani, J., and Hanahan, D.J. (1986) Characterization of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC)-induced protein phosphorylation in rabbit platelets: inhibitory effect of AGEPC analogs. *Arch. Biochem. Biophys.* **246**, 855-864.

Sullivan, J.M. (1984) Cardiovascular disorders and platelet function: An introduction. *In* "Blood platelet function and medicinal chemistry" (A. Lasslo, ed.) Elsevier Biomedical, New York, p1-11.

Swanson, S.J., Lin, B.F., Mullenix, M.C., and Mortensen, R.F. (1991) A synthetic peptide corresponding to the phosphorylcholine (PC)-binding region of human C-reactive protein possesses the TEPC-15 myeloma PC-idiotype. *J. Immunol.* **146**, 1596-1601.

Sweatt, J.D., Connolly, T.M., Cragoe, E.J., and Limbird, L.E. (1986) Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. *J. Biol. Chem.* **261**, 8667-8673.

Taki, T., and Kanfer, J.N. (1979) Partial purification of a rat brain phospholipase D. *J. Biol. Chem.* **254**, 9761-9765.

Tatsumi, N., Hashimoto, K., Okuda, K., and Kyougoku,T. (1988) Neutrophil chemiluminescence induced by platelet activating factor and suppressed by C-reactive protein. *Clin. Chim. Acta* **172**, 85-92.

Tebo, J.M., and Mortensen, R.F. (1990) Characterization and isolation of a C-reactive protein receptor from the human monocytic cell line U-937. *J. Immunol.* **144**, 231-238.

Tettenborn, C.S. and Mueller, G.C. (1988) 12-O-tetradecanoylphorbol-13-acetate activates phosphatidylethanol and phosphatidylglycerol synthesis by phospholipase D in cell lysates. *Biochem. Biophys. Res. Comm.* **155**, 249-255.

Tillet, W.S., and Francis, T. (1930) Serological reactions in pneumonia with nonprotein somatic fraction of pneumococcus. *J. Exp. Med.* **52**, 561-571.

Touqui, L., Rothhut, B., Shaw, A.M., Frodin, A., Vargaftig, B.B., and Russo-Marie, F. (1986) Platelet activation-a role for a 40k anti-phospholipase A_2 protein indistinguishable from lipocortin. *Nature* **321**, 177-180.

Tremblay, J., and Hamet, P. (1987) Cyclic nucleotides and calcium in platelets. *In* "Platelets in biology and pathology III", (MacIntyre, D.E. and Gordon, J.L., eds.) Elsevier Science Publishers, Amsterdam, p433-465.

Trowbridge, E.A., Martin, J.F., and Slater, D.N. (1982) Evidence for a theory of physical fragmentation of megakaryocytes, implying that all platelets are produced in the pulmonary circulation. *Thromb. Res.* **28**, 461-475.

Tschopp, J., Schafer, S., Masson, D., Peitsch, M.C., and Heusser, C. (1989) Phosphorylcholine acts as a Ca^{2+}-dependent receptor molecule for lymphocyte perforin. *Nature* **337**, 272-274.

Tysnes, O.-B., Steen, V.M., Aarbakke, G.M. and Holmsen, H. (1991) Stimulation of human platelets with low concentrations of thrombin: Evidence for equimolar accumulation of inositol triphosphates and phosphatidic acid. *Int. J. Biochem.* **23**, 305-310.

Uhlenbruck, G., and Karduck, D. (1979) A comparison of "serum lectins". Tridacnin, eel serum and C-reactive protein. *Naturwissenschaften.* **66**, 524-520.

van Blitterswijk, W.J., Hilkmann, H., de Widt, J., and van der Bend, R.L. (1991) Phospholipid metabolism in bradykinin-stimulated human fibroblasts. I. Biphasic formation of diacylglycerol from phosphatidylinositol and phosphatidylcholine , controlled by protein kinase C. *J. Biol. Chem.* **266**, 10337-10343.

Van Der Meulen, J., and Haslam, R.J. (1990) Phorbol ester treatment of intact rabbit platelets greatly enhances both the basal and guanosine 5'-[γ-thio]triphosphate-stimulated phospholipase D activities of isolated platelet membranes. Physiological activation of phospholipase D may be secondary to activation to phospholipase C. *Biochem. J.* **271**, 693-700.

Vance, D.E., Trip, E.M., and Paddon, H.B. (1980) Poliovirus increases phosphatidylcholine biosynthesis in HeLa cells by stimulation of the rate limiting reaction catalyzed by CTP:phosphocholine cytidylyltransferase. *J. Biol. Chem.* **255**, 1064-1069.

Vargaftig, B.B., Chignard, M., Benveniste, J., Lefort, J., and Wal, F. (1981) Background and present status of research on platelet-activating factor (PAF-acether). *Ann. N.Y. Acad. Sci.* **370**, 119-137.

Vetter, M.L., Gewurz, H., and Baum, L.L. (1986) The effects of C-reactive protein on human cell-mediated cytotoxicity. *J. Leukoc. Biol.* **39**, 13-25.

Vigo, C. (1985) Effect of C-reactive protein on platelet-activating factor-induced platelet aggregation and membrane stabilization. *J. Biol. Chem.* **260**, 3418-3422.

Volanakis, J. E. (1982) Complement activation by C-reactive protein complexes. *Ann. N. Y. Acad. Sci.* **389**, 235-250.

Volanakis, J.E. and Kaplan, M.H. (1971) Specificity of C-reactive protein for choline phosphate residues of pneumococcal C-polysaccharide. *Proc. Soc. Exp. Biol. Med.* **136**, 612-614.

Volanakis, J.E. and Kaplan, M.H. (1974) Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes Requirement for human C1q. *J. Immunol.* **113**, 9-17.

Volanakis, J.E., and Narkates, A.J. (1981) Interaction of C-reactive protein with artificial phosphatidylcholine bilayers and complement. *J. Immunol.* **126**, 1820-1825.

Volanakis, J.E., and Wirtz, K.W.A. (1979) Interaction of C-reactive protein with artificial phosphatidylcholine bilayers. *Nature* **281**, 155-157.

Watson, S.P., McNally, J., Shipman, L.J., and Godfrey, P.P. (1988) The action of protein kinase inhibitor, staurosporine, on human platelets. Evidence against a regulatory role for protein kinase C in the formation of inositol bisphosphate by thrombin. *Biochem. J.* **249**, 345-350.

Wang, C.M., Nguyen, N.Y., Yonaha, K., Robey, F.A. and Liu, T.Y. (1982) Primary structure of rabbit C-reactive protein. *J. Biol. Chem.* **257**, 13610-13615.

Wang, P., Anthes, J.C., Siegel, M.I., Egan, R.W., and Billah, M.M. (1991) Existence of cytosolic phospholipase D. Identification and comparison with membrane-bound enzyme. *J. Biol. Chem.* **266**, 14877-14880.

Weiss, H.J. (1991) Von Willebrand factor and platelet function. *Prog. Vasc. Biol. Hemost. Thromb.* **614**, 125-137.

Weiss, H.L., and Aledort, L.M. (1967) Impaired platelet/connective-tissue reaction in man after aspirin ingestion. *Lancet* **2**, 495-497.

Weissmann, G. (1991) Aspirin. *Sci. Amer.* **Jan.**, 84-90.

Werner, M.H., Bielawska, A.E., and Hannun, Y.A. (1991a) Quantitative analysis of diacylglycerol second messengers in human platelets: correlation with aggregation and secretion. *Mol. Pharmacol.* **41**, 382-386.

Werner, M.H., Bielawska, A.E., and Hannun, Y.A. (1992) Multiphasic generation of diacylglycerol in thrombin-activated human platelets. *Biochem. J.* **282**, 815-820.

Werner, M.H., Senzel, L., Bielawska, A., Khan, W., and Hannun, Y.A. (1991b) Diacylglycerol overcomes aspirin inhibition of platelets: evidence for a necessary role for diacylglycerol accumulation in platelet activation. *Mol. Pharmacol.* **39**, 547-556.

Whisler, R.L., Newhouse, Y.G., and Mortensen, R.F. (1983) C-reactive protein-(CRP) mediated modulation of human B-cell colony development. *J. Immunol.* **130**, 248-253.

Whisler, R.L., Newhouse, Y.G., and Mortensen, R.F. (1986a) C-reactive protein reduces the promotion of human ß-cell colony formation by auto-reactive T4 cells and T-cell proliferation during the autologous mixed lymphocyte reaction. *Cell. Immunol.* **102**, 287-298.

Whisler, R.L., Proctor, V.K., Downs, E.C., and Mortensen, R.F. (1986) Modulation of human monocyte chemotaxis and procoagulant activity by human C-reactive protein (CRP). *Lymphokine Res.* **5**, 223-228.

White, J.G. (1984) The ultrastructural and regulatory mechanisms of blood platelets. *In* "Blood platelet function and medicinal chemistry" (Lasslo, A., ed.) Elsevier Biomedical., New York, 15-54.

White, J.G. (1987a) An overview of platelet structural physiology. *Scanning Microsc.* **1**, 1677-1700.

White, J.G. (1987b) Views of the platelet cytoskeleton at rest and at work. *Ann. N.Y. Acad. Sci.* **509**, 156-176.

Winkelhake, J.L., and Chang, R.J., (1982) Acute phase C-reactive protein-like macromolecules from rainbow trout (*Salmo gairdneri*). *Dev. Comp. Immunol.* **6**, 481-490.

Wolf, R.A., and Gross, R.W. (1985) Identification of neutral active phospholipase C which hydrolyses choline glycerolipids and plasmalogen selective phospholipase A_2 in canine myocardium. *J. Biol. Chem.* **260**, 7295-7303.

Wood, H.F. (1951) Effects of C-reactive protein on normal human leukocytes. *Proc. Soc. Exp. Med. Biol.* **76,** 843-847.

Wood, H.F, McCarty, M., and Slater, R.J. (1954) The occurrence during acute infections of a protein not normally present in the blood. V. Physical-chemical properties of the C-reactive protein crystallized by a modified technique. *J. Exp. Med.* **100**, 71-79.

Wong, T., Pedvis, L., and Frujmovic, M.M. (1989) Platelet size affects both micro- and macro-aggregation: contributions of platelet number, volume fraction and cell surface. *Thromb. Haemostas.* **62**, 733-741.

Wright, T.M., Rangan, L.A., Shin, H.S., and Raben, D.M (1988) Kinetic analysis of 1,2-diacylglycerol mass levels in cultured fibroblasts. Comparison of stimulation by α-thrombin and epidermal growth factor. *J. Biol. Chem.* **263**, 9374-9380.

Yamada, K., Iwahashi, K., and Kase, H. (1987) K252a, A new inhibitor of protein kinase C, concomitantly inhibits 40 k protein phosphorylation and serotonin in phorbol ester-stimulated platelets. *Biochem. Biophys. Res. Comm.* **144**, 35-40.

Yang, C., Mookerjea, S., and Nagpurkar, A. (1992) Clearance of rat C-reactive protein *in vivo* and by perfused liver. *Glycobiology* **2**, 41-48.

Young, N.M., and Williams, R.E. (1978) Comparison of the secondary structures and binding sites of C-reactive protein and the phosphorylcholine-binding murine myeloma proteins. *J. Immunol.* **121**, 1893-1898.

Zahedi, K., Tebo, J.M., Siripont, J., Klimo, G.F., and Mortensen, R.F. (1989) Binding of human C-reactive protein to mouse macrophages is mediated by distinct receptors. *J. Immunol.* **142**, 2384-2392.

Zeller, J.M., Kubak, B.M., and Gewurz, H. (1989) Binding sites for C-reactive protein on human monocytes are distinct from IgG Fc receptors. *Immunology.* **67**, 51-55.

Zucker, M.B. (1980) The functioning of blood platelets. *Sci. Am.* **242**, 86-103.