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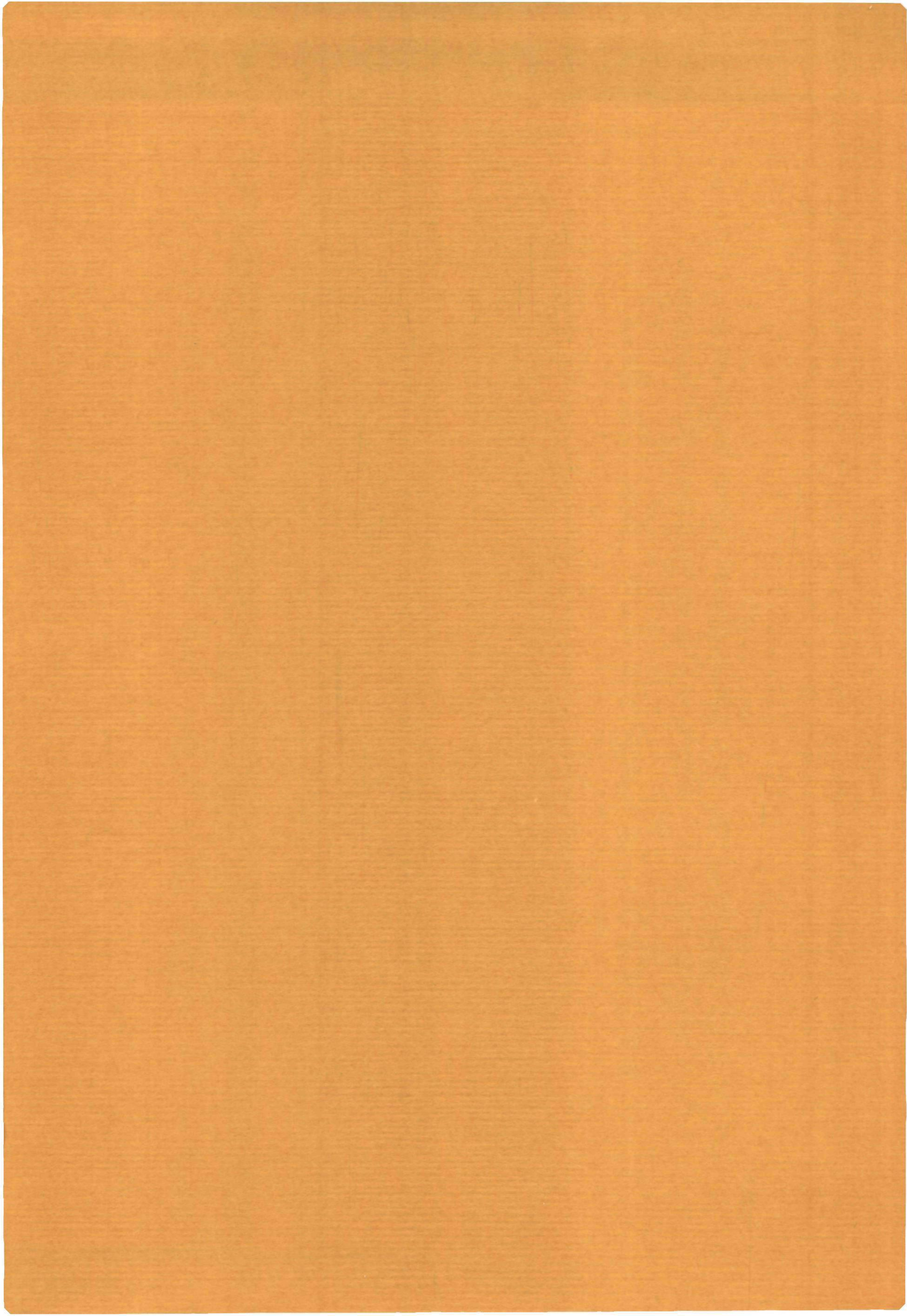
LITHIUM AND BLOODPLATELETS

LEON IMANDT



LITHIUM AND BLOODPLATELETS

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Aan mijn ouders

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CHAPTER 1

FUNCTION AND BIOCHEMISTRY OF BLOOD PLATELETS

INTRODUCTION

Platelets show in spite of their tiny size and absence of a nucleus the characteristics of a variety of secretory and excitatory cells. The ability of platelets to adhere to surfaces and to form aggregates is the basis of their physiological function: their contribution to normal hemostasis and maintenance of endothelial integrity. They also play a role in the pathogenesis of thrombosis, atherosclerosis and transplantation rejection (1).

1.1. PHYSIOLOGY AND FUNCTION

1.1.1. Platelet Structure

The use of electron microscopy has much revealed about the platelet structure (2). The data are compiled in Figure 1.
Platelets contain no nucleus and no DNA. Their membrane is trilaminary and contains many proteins, particularly glycoproteins.
Platelets contain a quantity of subcellular organelles and structures. The most important are:

- Mitochondria: organelles containing structures and enzymes for the Krebs cycle.
- α-Granules : organelles accumulating a.o. heparinneutralising protein (platelet factor 4), β-thromboglobulin, a growthfactor and mucopolysaccharides (3).
- Dense bodies: the most condense of all subcellular structures. They store vasoactive amines (such as serotonin), adenine-nucleotides (ATP and ADP) and calcium (3).
- Lysosomes : vesicles perhaps identical to α-granules, which contain acid hydrolases like acid phosphatase, beta-glucuronidase and cathepsin.
- Microtubules: tubular filaments in the equatorial plane of the platelets directly under the cytoplasmatic membrane. Very probably the microtubules have contractile properties (4). It has been shown

that depending on the functional situation the microtubules in the platelets are present or absent, which suggests that they can be built up or broken down very quickly.

- Membrane-systems : the so called "Dense Tubular System" and the "Surface Connected Canalicular System".
The Dense Tubular System is visible in E.M. as tubular structures, which are probably comparable to the sarcoplasmatic reticulum in striated muscles.
The Surface Connected Canalicular System is built up of a larger tubular system and is in fact produced by intrusions of the platelet outer membrane. The content of the Surface Connected Canalicular System is for this reason in open connection with the platelet surrounding bloodplasma.

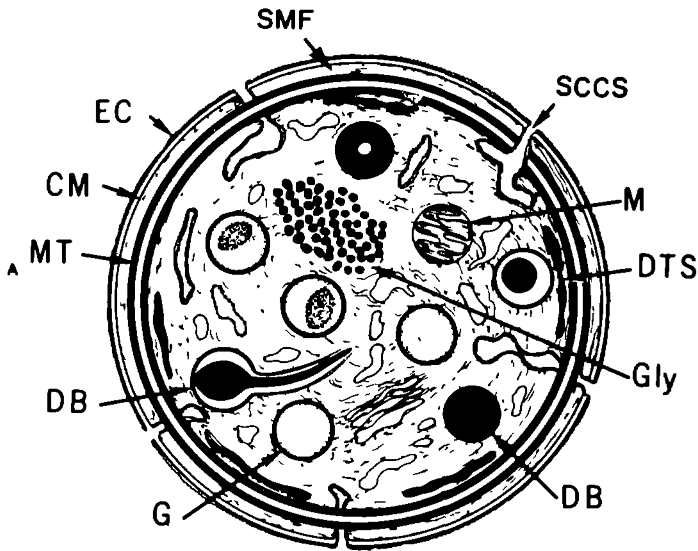
1.1.2. Platelet Function

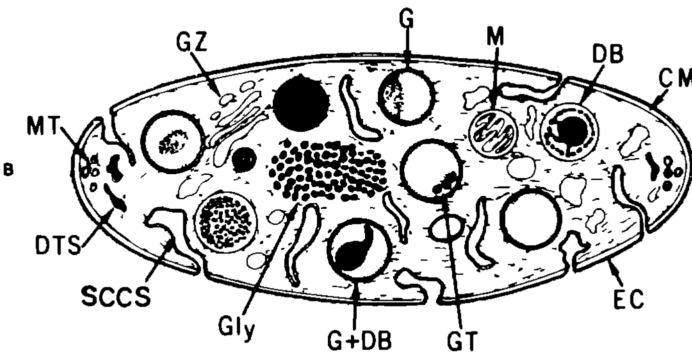
Platelet adhesiveness

When platelets come into contact with foreign surfaces they stick to it (adhesion). During adhesion a spreading of the platelets occurs in which process pseudopods are formed (5,6). Directly after damage of a vessel platelets adhere onto the underlying structures like collagen, microfibrils and basement membrane.

Platelet "shape change"

As a result of the stimulus which induces adhesion or aggregation a shape change of platelets occurs. The disc-like form changes into a "spiny-sphere" and the microtubules disappear, possibly because of contraction or because of desintegration. After the "shape change" other changes in morphology occur like the formation of pseudopods and swelling. Besides, "release" starts. These last mentioned changes are not reversible any more and belong as such not to the essentially reversible shape change.





TEXT-FIGURE 1A and B—Diagramatic representations of blood platelets as they appear in thin sections by electron microscopy a platelet cut in the equatorial plane (A) and a platelet in cross section (B) Components of the peripheral zone include the exterior coat (*EC*) trilaminar unit membrane (*CM*) and submembrane area (*SMF*) which form the wall of the platelet and line the channels of the surface connected open canalicular system (*SCCS*) The matrix of the platelet is the sol-gel zone containing microfilaments submembrane filaments (*SMF*) the circumferential band of microtubules (*MT*) and glycogen (*Gly*) Formed elements embedded in the sol-gel zone include mitochondria (*M*) granules (*G*) dense bodies (*DB*) and channels of the dense tubular system (*DTS*) Collectively they constitute the organelle zone A Golgi apparatus (*GZ*) is found in occasional platelets

FIGURE 1

Structure of blood platelets

Reprinted with permission from: J.White and J.Gerrard; Am.Journ.of Pathology 83, 591, 1976.

Platelet "release" reaction

Induced by various events (see under platelet aggregation) and during platelet adhesion an expulsion occurs of organelles containing enzymes, nucleotides and ions. This phenomenon is called the "release" reaction (7). Electron microscopy revealed that the release reaction is caused by a contraction of the microtubules. During contraction the organelles of the platelet are moved towards the centre. The content of the dense bodies (release I) and of the α-granules (release II) are pressed into the Surface Connected Canalicular System and in this way expelled out of the platelet.

Platelet aggregation

Adhesion of platelets to foreign surfaces is accompanied by sticking of free platelets passing by in the blood stream to the already adhered platelets and to each other. This process is called platelet aggregation (8). Platelet aggregation can be induced by various stimuli and requires extracellular calcium. A lot of knowledge has been obtained about the aggregation process by in vitro studies. When very small quantities of aggregation inducers are added to a platelet suspension under stirring then the occurring aggregation appears to be completely reversible and after a certain time the aggregates disperse again. This reversible phase of aggregation is called the first phase. At higher concentrations of the stimulus the aggregation becomes irreversible, that means that the first phase of aggregation is followed by a second phase. In general the second phase is caused by a release of ADP which at a certain level becomes so intensive that it forms as such a stimulus for further aggregation by which a snowballeffect of aggregation occurs (7). However, recently it has been demonstrated (9) that a causal relation between release and "second phase" aggregation does not hold for all inducers.

Platelet factor "availability"

It has been found that stimules which cause aggregation bring about changes in the platelet membrane (10). During these membrane changes not only the electrostatic properties of the

platelet change (11) but also structures and receptors come available which are normally not accessible. This process is called the "availability reaction". One of the most important structures that become available are phospholipids (platelet factor 3) which at their surface stimulate the interaction of coagulation factors (12).

Platelet fusion and contraction

The exposion of phospholipids together with the temporary standstill of the bloodstream within a platelet clump gives the coagulation mechanism the opportunity to interact, resulting in the formation of traces of thrombin.
Thrombin causes a complete release of the platelet content and the platelets melt to an irreversible packed mass in which no individual cells can be recognized. After platelet fusion spontaneous contraction of the platelet aggregate occurs. This phenomenon is well known as retraction. This contraction points to the existence of a contractile system that controls platelet shape, governs extrusions and contractions of pseudopods, and causes release.

In conclusion it can be stated that the special function of platelets in the coagulatory mechanism is, besides the delivering of phospholipids, especially to shape time for interaction of coagulation factors within the platelet aggregate.

1.2. PLATELET BIOCHEMISTRY

1.2.1. Introduction

It is very remarkable that aggregation and release reaction of platelets can be induced by so many different substances. This implies that the biochemical processes (13,14) within the platelets are more important than the physical or chemical type of the stimulus.
It was formerly thought that in all cases the induction of aggregation proceeds through release of ADP and consecutive ADP-aggregation (15). On this supposition a number of theories for

aggregation were based, the theory concerning inhibition of an ecto-ATPase by ADP being the most important (16,17). The current view is that aggregation occurs after induction of the release reaction, formation of prostaglandins or increase of the cytoplasmic calciumconcentration. In normal platelets the three phenomena act synergistically and are closely related (18). The magnitude of aggregation is modulated by the level of cyclic AMP. It is therefore difficult to study the effect of a certain stimulus specifically on one of the separate pathways.
Prostaglandin synthesis and intracellular calcium are together with the level of cyclic AMP intracellular regulatory mechanisms that control platelet function. The release reaction may occur as a secondary effect of intracellular changes.
Many of the aggregation inducing substances do not enter the cell, but stimulate the platelets via interaction with the membrane. Obviously the relation between membrane reactions and intracellular metabolic processes is very important.
In the next sections various aggregation inducers and the intracellular regulation mechanisms are discussed.

1.2.2. Platelet Aggregation Inducers

All kinds of stimuli which induce aggregation also cause "shape change" and "platelet-factor 3-availability" and if strong enough "release reaction". The following substances are known to be aggregation inducers:

Adenosine Diphosphate (ADP)
ADP is a very specific inducer of platelet aggregation. This specific action of ADP was first shown by Gaarder et al. in 1961 (19). ADP induces aggregation in concentrations of 10^{-7}M to 10^{-5}M but only if traces of calcium and fibrinogen are present (16,20). Without calcium present no aggregation occurs but only shape change. ADP in concentrations of more than 10^{-6}M can induce release and irreversible platelet aggregation.
ADP as such cannot enter the cell and for this reason a specific ADP receptor was supposed to be present on the platelet membrane. Studies of Nachman and Ferris (21,22) indeed suggest that ADP

binds to a specific receptor. Very recently Bennett et al. (23) reported the binding of ADP to 4 proteins in platelet homogenates, of which one polypeptide also binds ADP in intact platelets. This polypeptide probably represents the ADP receptor. The exact mechanism of ADP-induced aggregation is not known. A few enzymatic theories have been developed, such as the inhibition of a platelet ecto-ATPase (16,17). Recently another membrane enzyme, nucleotide diphosphokinase, has been proposed as a possible mediator of ADP induced aggregation (24,25). This enzyme catalyses the transfer of a terminal phosphate of ATP to ADP. Substances that diminish the activity of this enzyme came out to be also inhibitors of platelet aggregation.
Till now no theory has gained general acceptance.

Prostaglandins

Prostaglandins play an important role in the aggregation process. The relevance of stable prostaglandins for platelet function was already recognized years ago. In the last years it has been shown that platelets themselves produce unstable metabolites of arachidonic acid. Aggregation inducers stimulate specifically the liberation of arachidonate from the platelet membrane (26, 27). The platelet can metabolize arachidonate to the very strong aggregation inducers endoperoxides (28-31) and thromboxane A_2 (32). The inhibition of the synthesis of these metabolites by drugs like aspirin completely abolish the release reaction and second phase aggregation (33,34). The importance of the prostaglandin system has been extended by the recent discovery of a very strong aggregation inhibitor, prostacyclin or PGI_2, which is synthesized in the endothelial cells of the vessel wall from endoperoxides (35,36). The mechanisms of action of prostaglandins will be discussed in more detail under the heading "Intracellular Regulatory Mechanisms".

Collagen

Collagen induces very specifically a strong release reaction when platelets adhere to it. The released ADP gives rise to an enhancement of the aggregation (37).

Chiang and coworkers (38) have reported that platelets contain a specific collagen receptor that might be the enzyme glycosyltransferase (39). This supposition is in accordance with the concept that adhesion is based on the formation of an enzyme-substrate complex between carbohydrate residues of collagen and the enzyme on the platelet membrane (40, 41). However, some doubt has been thrown upon this concept (42-44).

Ionophores

Ionophores are antibiotics that make membrane permeable to cations. The ionophores A23187 and X537A are specific for calcium (45,46). In low concentrations the ionophore A23187 induces the same morphological changes, aggregation and release as other aggregation inducers (47). In high concentrations it causes destruction of the platelet (48,49). The former idea that ionophore aggregates platelets only via the release-reaction (50) has been rejected since thrombin-treated platelets (without releasable pool) can still be aggregated by ionophore (51). The mechanism of action is discussed in more details under the heading "Intracellular Regulatory Mechanisms".

Other aggregation inducers

Besides ADP, arachidonate metabolites, collagen and ionophores that are used in this study, there are other aggregation inducers. The most important are thrombin, adrenalin, serotonin and ristocetin (see for reviews: 10,14).

1.2.3. Intracellular Regulatory Mechanisms

A. Regulatory Role of Calcium

Calcium plays an important regulatory role in many cells (52), particularly in muscle cells (53) and secretory cells (54). The similarities between platelets and these cells have led to many studies on the relationship between calcium and platelet release.

Research on the role of calcium in platelet aggregation is hampered by the facts that extracellular calcium ions are essential for induction of aggregation (55) and that the con-

centration of calcium within the various platelet compartments varies considerably (56).
There are, however, strong indications that intracellular calciumions play an essential role in platelet stimulus-response coupling (57):
1. Platelets are contractory cells like muscle cells and with secretory properties like endocrine cells, leukocytes and mast-cells (58). In all these cells calcium regulates the contractile function of the cells.
2. As described under "Aggregation Inducers", ionophores can induce aggregation and release in platelets by raising the intracellular calciumconcentration. The ionophore A23187 induces release, even when no extracellular calcium is present (50, 59,60). This indicates that intracellular liberation of calcium is sufficient for release induction (60). When extracellular calcium is present the ionophore causes uptake of calcium (48, 50,60) and aggregation occurs.
3. The third piece of evidence for a role of calcium is more direct. Drugs that inhibit intracellular calcium fluxes also inhibit aggregation e.g. D_2O and chlorotetracycline (62), local anesthetics such as dibucain and tetracain (63) and other synthetic compounds (64,65). Also other indications for calcium redistribution during aggregation have been reported (66,67).
These three pieces of evidence are indications that intracellular calcium regulates platelet function. The origin of the calcium which raises the cytoplasmatic calcium concentration can either be extracellular, from transport into the cell, or intracellular, being made available by calcium redistribution.
There are four types of calciumfluxes in platelets (68):
1. Calciumions are liberated from membranes, particularly for shape change. This pool is easily exchangeable.
2. Liberation of calcium from vesicular storage organelles into the cytoplasm, inducing the release reaction as proposed by White (60).
3. Release from the storage organelles (dense bodies) directly into the extracellular fluid (69).
4. A rearrangement of the plasmamembrane during release results in an increased amount of calcium binding sites and calcium

exchange.

This last mentioned calciumflux is more a result than a cause of platelet stimulation (70-72).

Most important in the regulatory role of calcium might be the calcium flux out off the storage vesicles of platelets. Such a membrane vesicle system, comparable to the sarcoplasmic reticulum ("relaxing factor") of muscle, has been demonstrated in blood platelets (73-75). It has been localised in the Dense Tubular System as an ATP dependent and cyclic AMP-stimulated active calciumtransport system (76-78). Käser-Glanzman et al. (76,78) have also concluded that the same Dense Tubular System plays a key role in transporting calcium out of the cytoplasm. Ionophores would mainly facilitate the intracellular calciumfluxes from these vesicular pools.

The mechanism by which calcium exerts its effects as an intracellular messenger in platelets probably resembles the mechanism in other cells.

It influences especially the contractile proteins of cells, partly via regulation of phosphorylation and dephosphorylation reactions. In platelets calcium affects the contractile protein thrombosthenin and the microtubule proteins (2,79). These proteins are extremely important in maintaining the shape of platelets at rest, changing it upon stimulation and in the release reaction.

In conclusion, calcium is essential for platelet function, inside and outside the platelets. Extracellularly it is indispensable for aggregation, although not for release induction by inducers that directly influence the intracellular calcium. Intracellularly it may well be the basic platelet regulator, as suggested by Holmsen (80).

B. Regulatory Role of Cyclic Nucleotides

Cyclic Nucleotides in general

The pioneerwork of Sutherland and coworkers (81) has shown that many hormones ("first messengers") in a variety of cells act through the intracellular messenger cyclic AMP ("second messenger"). Cyclic AMP is important in many cell- and tissue-functions,

for instance the energy metabolism (82,83), growth and differentiation (84) and transmission of signals (85,86). Cyclic AMP is formed from ATP by the enzyme adenylate cyclase and degraded to AMP by phosphodiesterase. Adenylate cyclase is influenced a.o. by hormones via specific receptors on the membrane of the cell. These receptors are in some way coupled to the enzyme but the exact mechanism is not known (87).
Another cyclic nucleotide is cyclic GMP, which is synthesized by guanylate cyclase and broken down by specific phosphodiesterases. It is found in many cells but the physiological function of this nucleotide is much less clear.

Enzymes of the platelet cyclic nucleotide system

The enzymes of the platelet cyclic nucleotide system are not well characterized. Adenylate cyclase has many features in common with adenylate cyclases of other cells, such as stimulation by fluoride, PGE_1 and glucagon (88-90) and a need for GTP (91, 92). The enzyme is membrane bound for the largest part (93,94). Calcium is a strong inhibitor of its activity (89,94), while magnesium is essential for its activity.
The platelet guanylate cyclase is for more than 95 % present in soluble form in the cytoplasmatic compartment (95,96) and has been isolated by chromatography (97). It needs Mn^{2+} for optimal activity, while Mg^{2+} and Ca^{2+} stimulate it slightly in the presence of Mn^{2+}.
Platelets contain at least three enzymatically different phosphosdiesterases (98-100), which are all exclusively located in the cytosol (94). One of the phosphodiesterases is a more specific cGMP phosphodiesterase, one a more specific cAMP phosphodiesterase and the third is non-specific.

Cyclic AMP and platelet function

After the first report on cAMP and platelet function in 1965 (101), it was four years before the research really started. Since then an enormous amount of reports has been published about this subject as well as a number of reviews (13,102-104). The general consensus is that an increase in the cAMP concentration inhibits platelet function. But whether the reverse holds

true, that is that aggregation inducers lower cAMP content, is a matter of much controversy.
The effects of drugs and aggregation inducers on cAMP metabolism have mostly been studied in lysed cell preparations.
The essential question is, however, whether changes in cAMP concentration or metabolism in intact platelets are related to platelet function and vice versa. The study of this question is hampered by the imperfections of the methods available (105-107).
Salzman reported that isolation of platelets (by centrifugation or gelfiltration) induces changes in the cAMP metabolism (108, 109).
Measurement of platelet cAMP in platelet-rich plasma is difficult since more than 75 % of the total cAMP in PRP samples originates from the plasma. Moreover, the cAMP in platelets is stored in more than one compartment (110,111). Since ATP cannot enter the cell, it is necessary to use labeled adenine as a precursor for ATP in studying the synthesis or degradation of cAMP in intact cells (either adenylate cyclase or phosphodiesterase activity). The very low conversion of labeled adenine into labeled cAMP in intact platelets (0.03 % - 0.1 % (104)) poses tremendous technical problems.
Cyclic AMP and dibutyril-cyclic AMP inhibit in vitro adhesion (112), aggregation, release (113,114) and clot retraction (115). They also show similar effects in vivo (116).
Adenylate cyclase activators and phosphodiesterase inhibitors all inhibit platelet function. The adenylate cyclase activators include some prostaglandins such as PGE_1 and prostacyclin (35, 117-121), adenosine and its 2-substituted derivates (122) and β-adrenergic agonists (123). Phosphodiesterase inhibitors are mostly used for fortifying the effects of adenylate cyclase activators. The most important phosphodiesterase inhibitors are theophyllin, papaverin and dipyridamole (124).
Recently the inhibitory action of some drugs on platelet adenylate cyclase have been reported. These drugs are synthetic analogues of deoxyadenosine, such as 2,5 di-deoxyadenosine and 9-(tetrahydro-2 furyl) adenine (called SQ 22536) (125-127).
SQ 22536 antagonizes the effects of substances that stimulate platelet adenylate cyclase, such as PGE_1, PGI_2 and adenosine.

Also the basal, unstimulated adenylate cyclase activity is inhibited by SQ 22536 and 2,5 di-deoxyadenosine (126) and Salzman reported even a lowering of the platelet cAMP concentration (123). However, there is no agreement on the effect of these compounds on platelet aggregation. Haslam reported that they do not induce aggregation themselves and have no effect on aggregation induced by known inducers (126,127). Salzman reported a stimulation by SQ 22536 of the prostaglandin endoperoxide induced aggregation but no consistent effect on aggregation induced by ADP and other agonists (125).
These results show that lowering of platelet cyclic AMP or adenylate cyclase activity is not enough for inducing aggregation. It is possible that only a fraction of the platelet cAMP regulates platelet function and that this fraction is not influenced by the adenosine analogues. This would be in accordance with the lack of correlation between the cAMP level and the amount of aggregation (128).

Aggregating agents and cyclic AMP

Perhaps the most controversial issue in the research of the last years on platelets is the question whether aggregating agents lower adenylate cyclase activity and/or cAMP content of platelets, or even induce aggregation through this inhibition. The results with almost all inducers are consistent with regard to inhibition of stimulated adenylate cyclase and the accumulation of cAMP, but the reports on effects on basal adenylate cyclase are contradictory. Such contradictory results have been reported for ADP, serotonin, adrenalin and noradrenalin (see reviews 13, 102-104) and also for the most recently discovered aggregation inducers, the prostaglandin endoperoxides and thromboxane A_2 (129-133).
Haslam measured basal adenylate cyclase activity by determining the rise in cAMP during inhibition of phosphodiesterase (104). He found that various aggregating agents show opposite effects on basal adenylate cyclase activity: ADP inhibited the enzyme, but adrenaline stimulated it and noradrenaline, serotonin and vasopressin had no effect.

Aggregating agents and cyclic GMP

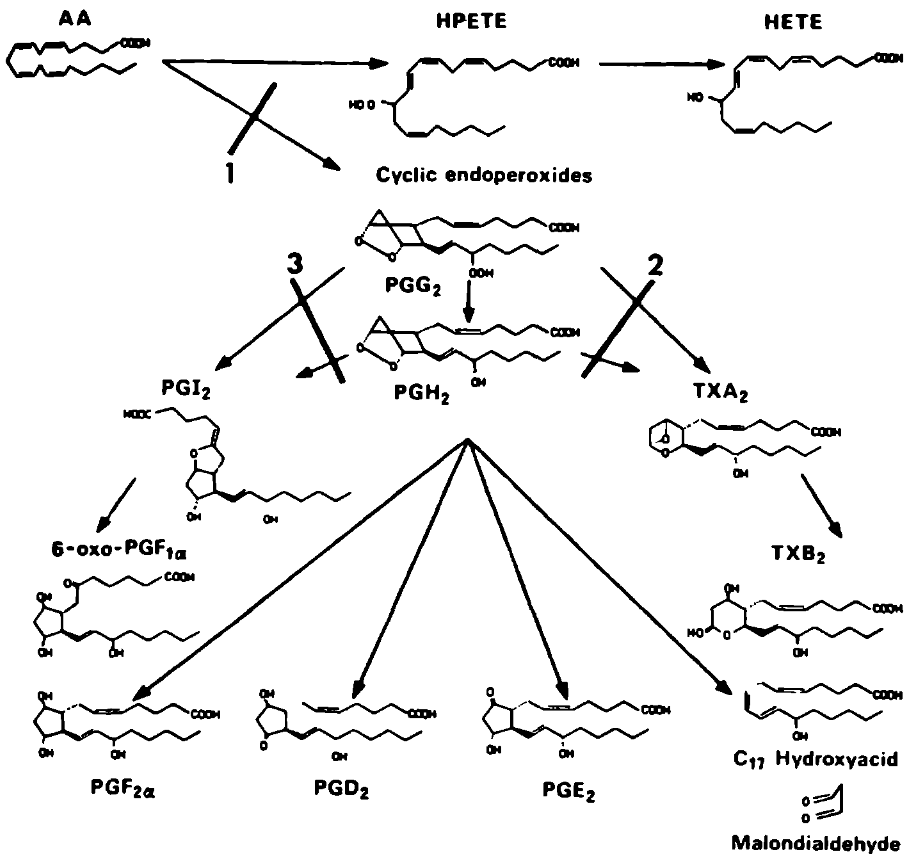
Almost all aggregation inducers, e.g. collagen, adrenalin, serotonin and arachidonate and its metabolites cause an increase in cGMP levels (94,104,134,135). The meaning of this increase is unknown since ascorbic acid and fatty acids other than arachidonic acid also raise the cGMP level without effect on aggregation (94,134,136,137).

Ways of action of cyclic AMP

Cyclic AMP exerts its effects on cellular functions mainly through regulation of phosphorylation of proteins by kinases and phosphorylases. Platelets contain protein kinases that do not differ from those of other cells (138-144). Part of these enzymes are probably regulated by cAMP and part are cAMP independent but calcium regulated (145). Three or four main proteins are phosphorylated (140,145).
Dibutyryl-cAMP inhibits the stimulation of phosphorylation of two of these proteins during aggregation (143,146). However, the physiological significance is not yet clear (142,146,147). Theories for regulation of platelet function by phosphorylation/dephosphorylation reactions have been put forward by Booyse (139) and Assaf (148) but they are highly speculative.
cAMP does influence the two other regulatory systems. It inhibits the membrane phospholipase activity (149) and lowers the availability of arachidonate for cyclooxygenase (150,151). Besides this, cAMP also stimulates a calcium pump (76). In this way cAMP might promote compartmentalization of calcium and inhibit the phospholipase activity (152,153).

C. Regulatory Role of Prostaglandins

Platelets synthesize stable prostaglandins (154-156). The research on prostaglandins in platelets received a new impetus with the discovery that drugs such as aspirin and indomethacin inhibit prostaglandin biosynthesis (157-159). The inhibition of this systhesis is accompanied by an inhibition of the platelet release reaction (33,34). Since the prostaglandin synthesis is enhanced during aggregation, particularly when induced by



Abbreviations:

AA: arachidonic acid	HPETE: 12-hydroxyperoxyarachidonic acid	TXA_2: thromboxane A_2
HETE: 12-hydroxyarachidonic acid	PGI_2: prostacyclin	TXB_2: thromboxane B_2

The sites where cyclo-oxygenase inhibitors (aspirin-like drugs), thromboxane synthetase inhibitors and prostacyclin synthetase inhibitors exert their action are indicated by the numerals 1, 2 and 3, respectively

FIGURE 2

Metabolic pathway of arachidonic acid

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arachidonic acid (160) it is clear that they play a role in platelet functionality.

Prostaglandin endoperoxides and thromboxanes

Stable prostaglandins do not induce aggregation. In the last years some unstable metabolites of arachidonic acid were found to induce aggregation: the prostaglandin endoperoxides (28-31) and the thromboxanes (32). (Fig. 2).
These metabolites are synthesized from arachidonate, which is incorporated in phosphatides of membranes (161) and liberated by a specific phospholipase A_2. Aggregation inducers stimulate specifically the liberation of arachidonate (26,27). The arachidonate can be metabolized in two ways: conversion through the enzyme lipoxygenase into the inactive compounds HPETE and HETE or conversion through the enzyme cyclooxygenase, that produces prostaglandin endoperoxides PGG_2 and PGH_2 (162,163), formerly called "Labile Aggregation Stimulatory Substance" or LASS (164). The endoperoxides, strong aggregation inducers and vasoconstrictors, are very unstable in aqueous solution ($t_{½}$ approx. 5 min) and are converted into stable prostaglandins or a 17-carbon hydroxyacid plus malondialdehyde. During aggregation the endoperoxides can be released (31,165).
In platelets the endoperoxides can be converted through the enzyme thromboxane synthetase into thromboxane A_2 (TxA_2), that is an even stronger aggregation inducer and vasoconstrictor (166-168). This substance is very unstable (166). Perhaps endoperoxides have to be converted into TxA_2 before they can induce aggregation (131,166,169-171). In aqueous media TxA_2 is converted into the stable thromboxane B_2, that has no effect on platelets.
During platelet stimulation also a burst in oxygen uptake is observed. This is partly a reflection of the prostaglandin endoperoxide synthesis (172).
The prostaglandin synthesizing system has been isolated from platelet microsomes (173) and is located in the Dense Tubular System (174). The two enzymes cyclooxygenase and thromboxane synthetase have been isolated and characterized (175-179).

Patients with a deficiency in cyclooxygenase (180,181) or thromboxane synthetase (181) have a mild bleeding tendency. Defects in lipoxygenase lead to an increased tendency to spontaneous aggregation (183) and a raised endoperoxide level has been noted in thrombosis (184). The effects of aspirin and other non-steroid anti-inflammatory drugs mimic enzyme deficiences. These drugs act through interference with cyclooxygenase (group one in Fig. 2) and induce a mild bleeding tendency (185-187). A second group of drugs, interfering with prostaglandin biosynthesis, are the thromboxane synthetase inhibitors. These include benzydamine, imidazole, endoperoxide analogues (173,188-193) and perhaps sulphinpyrazon (194).
How endoperoxides and TxA_2 induce aggregation is not known precisely (195). They do not aggregate by release induction only (196) since shape change and aggregation have been demonstrated without release occurring (197,198). They affect the stimulated adenylate cyclase but not the basal enzyme activity (129-133), just as other aggregation inducers.
A very intriguing suggestion has been made by Gerrard and White (175,199). They suppose that TxA_2, which is synthesized in the Dense Tubular System, is liberated into the cytoplasm and acts as a physiological ionophore by transporting calcium out of the Dense Tubular System into the cytoplasm (199,200). Indirect support for this hypothesis emerges from the observation that aggregation with ionophore and thrombin probably directly raises the cytoplasmatic calcium concentration and is independent of cyclooxygenase products (49).

Prostacyclin or PGI_2

The discovery of a specific vessel wall prostaglandin, prostacyclin or PGI_2 (35,36,201), has put the regulation of platelets in a new light. It is synthesized enzymatically from endoperoxides and is the strongest aggregation inhibitor and vasodilator discovered until now (35,202). It inhibits platelet adhesion to subendothelium (203) and the formation of platelet plugs (203,204) and even disperses platelet aggregates. Prostacyclin is very unstable in blood at $37^{\circ}C$ and is degraded to the relati-

vely inactive 6-oxo-$PGF_{2\alpha}$ (201). Fresh venous and arterial slices and microsomes of pig, rabbit, dog and man synthesize PGI_2 (205-209). The generation of prostacyclin by lungs (210, 211) has led to the proposal that prostacyclin is a circulating anti-coagulant (211,212). The site of production is the endothelial cell layer of the vessel wall (213,214). Also cultured endothelial cells produce PGI_2 (215). The conversion of arachidonate into PGI_2 amounts to no more than 1 %, while endoperoxides are converted to an extent of more than 80 % (35,36,205, 216,217). The synthesis of PGI_2 is inhibited by lipid peroxides (group 3 in Figure 2) (204,217).
Inhibition of the formation of lipid peroxides may be important for thrombosis prevention. The mechanism of action of prostacyclin is stimulation of the platelet adenylate cyclase by binding to a specific receptor (218). It raises thus the pla-

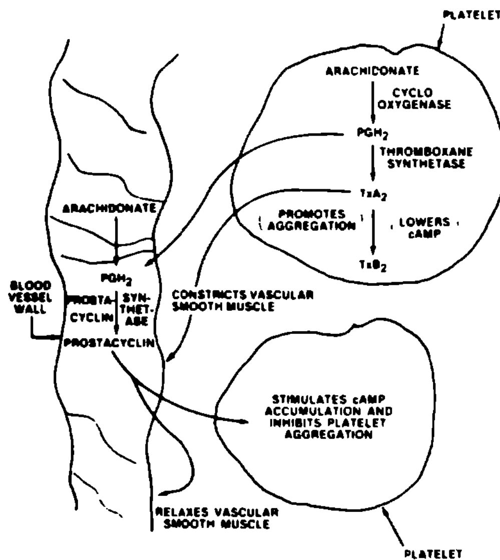

FIGURE 3

Model of human platelet homeostasis in vivo.
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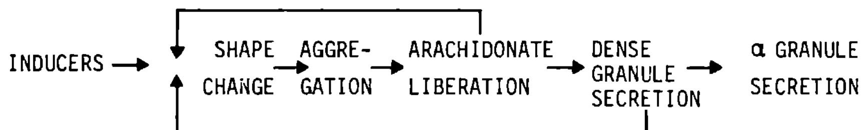
telet cAMP concentration (119-121), inhibiting also the phospholipase A_2 activity of the platelet membrane (149).
The discovery of prostacyclin has tremendous consequences for the understanding and research on platelet regulation in vivo. Endoperoxides are preferentially used as substrate by prostacyclin synthetase, and might be provided by the platelets. This leads to a very elegant scheme of platelet regulation in vivo (see Fig. 3) (120,121): stimulation of the platelets leads to formation of endoperoxides and TxA_2 with lowering of the platelet cAMP content.
However, the endoperoxide can enter the vessel wall and be converted into PGI_2, which raises the cAMP content in platelets. In vivo platelet function would be balanced by these opposing mechanisms. When the vessel wall is damaged or absent (in vitro) this regulation is out of balance and aggregation is promoted.
Although recent results (219,220) indicate that the endoperoxides are not easily exchanged between platelets and vessel wall, the clinical implications of the existence of partly similar enzyme systems in platelets and vessel wall are very important. Drugs that inhibit the cyclooxygenase of platelets, such as aspirin, also influence the vessel wall cyclooxygenase and PGI_2 production (221,222). Selective thromboxane synthetase inhibitors may be useful for thrombosis prevention (219).

D. Interrelationships between regulatory functions

At the moment it is not known precisely which mechanism regulates the responses of platelets to physiological stimuli. The interrelationships between the regulatory processes can be surveyed only by fitting together the separately observed processes and developing a theory on that basis.
Holmsen (223) has developed such a theory. He proposed that all platelet reactions are the consequence of one basic platelet function. This basic platelet mechanism would be elevation of the cytoplasmatic calciumconcentration (80). Platelets can respond in different ways to stimulation, depending on the strength and character of the stimules. Recently (80) he has

refined this theory and made a list of platelet reactions in order of increasing strength:



The arachidonate liberation, resulting in endoperoxide and thromboxane formation,and the release of ADP, serotonin and calcium would act as positive feedback loops. Most data known about the aggregation process can be fit into this hypothesis. Stimulation of platelets with ADP starts by binding of ADP to the platelet membrane with induction of shape change, then aggregation etc.

Kinlough-Rathbone et al. (18,224) showed that the arachidonate liberation and calcium elevation can be induced directly e.g. by thrombin or ionophores. This means that there are different starting points in the scheme of Holmsen, all resulting in the same consequences.aggregation and/or release.

In normal platelets the diverse regulation mechanisms influence each other.

The synthesis of TxA_2 can lead, possibly by functioning as a ionophore (114,199,200), to an elevation of the cytoplasmic calciumconcentration.

An elevation of the cytoplasmatic calciumconcentration in its turn stimulates the liberation of arachidonate via the enzyme phospholipase A_2 (225-227). As well prostaglandin endoperoxides/ TxA_2 as an elevated calciumconcentration inhibit platelet adenylate cyclase (95,129-133).

A lowering of platelet cyclic AMP is not sufficient for inducing aggregation (126,127). However, since an elevated cAMP level inhibits as well the liberation of arachidonate (76, 149-151) as possibly also the enzyme cyclooxygenase (228,229), low levels of cAMP probably promote aggregation.

Besides, elevated cAMP levels stimulate the removal of calcium out off the cytoplasm (152,153). Cyclic AMP might act as a negative feedback mechanism in platelet regulation.

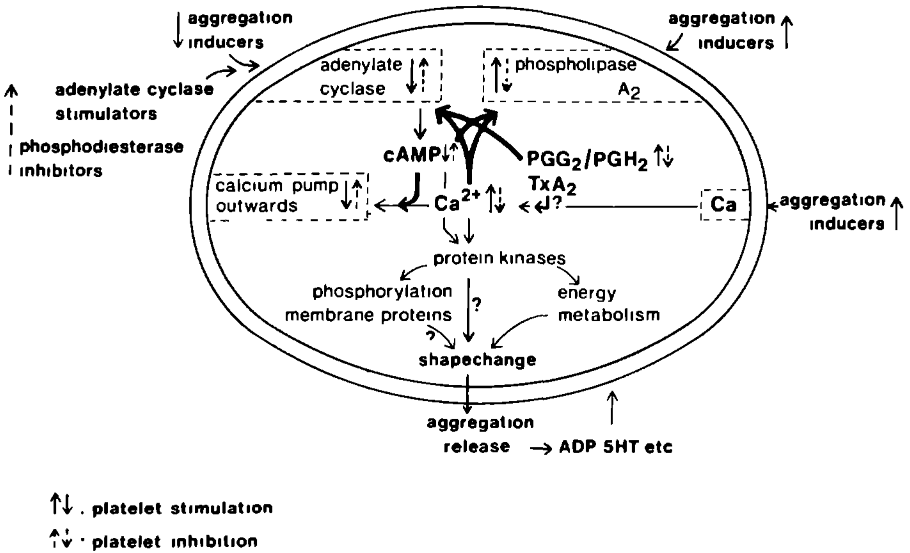


FIGURE 4

Interrelationships of platelet regulation mechanisms

↑ : stimulation of enzymes or elevation of metabolites by aggregation inducers.

↓ : inhibition of enzymes or decrease of metabolites by aggregation inducers.

⇡ : stimulation of enzymes or elevation of metabolites by aggregation inhibitors.

⇣ : inhibitors of enzymes or decrease of metabolites by aggregation inhibitors.

These interrelationships lead to a network of feedback mechanisms (see fig. 4), which can explain the perfect control system that is needed for the very subtle regulation of platelet function.

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CHAPTER 2

LITHIUM: CHEMISTRY, BIOCHEMISTRY AND PHARMACOLOGY

INTRODUCTION

Lithium has important biological actions, which is demonstrated by its therapeutical effect in some mental illnesses for which it is in use as a potent drug in psychiatric practice since 1949 (1,2). The mode of action of lithium on cellular function and biochemical or metabolic processes is unknown. A clarification of the mechanism of its action on platelet metabolism may offer an important lead for the elucidation of its action on the functionality of other cells. Knowledge about the mechanism of its action could also be useful for understanding the normal regulatory mechanisms of platelets. The key to the solution of the mechanism of the action of lithium could lie in its special chemical and biological properties (3).

2.1 CHEMISTRY OF LITHIUM

Lithium is the smallest of the alkali metals and shows a strong tendency to form monovalent cations (4). Lithium has much in common with sodium and potassium but surprisingly it also displays properties of the bivalent earth alkali metals e.g. of calcium and magnesium. This is demonstrated by its binding constant for complex anions (e.g. EDTA) which lies between those for Na/K and Ca/Mg. The explanation of this hybridic behaviour is given by the specific physical properties of lithium: the ionradius of Li^{+} (0.60Å) is almost equal to that of Mg^{2+} (0.64 Å) while the charge density (0.22 Coul/Å^2) resembles that of Ca^{2+} (0.16 Coul/Å^2). The physical properties of the other alkalimetals show a much greater difference to those of the earth-alkali metals: Na^{+} and K^{+} have a ionradius of 0.95 Å and 1.33 Å and a charge density of 0.088 and 0.045 Coul/Å^2 respectively. These physical properties of lithium could possibly explain many of its biological effects as suggested by Frausto da Silva et al. (5): lithium may substitute in ligand formation in vivo for Ca/Mg though the affinity of Ca/Mg for natural ligands is greater than that of lithium, since in therapeutic circumstances the concentration of li-

thium is higher than that of free calcium and magnesium.

2.2 PHARMACOLOGY OF LITHIUM

In normal conditions the concentration of lithium in animal tissues is extremely low. The concentration in the blood at which therapeutic effects may be obtained lies between 0.5 and 1 mmol/l plasma (6). The effective concentration range is very narrow since at values above 2 mM toxic side effects occur.
Many investigations on the effects of lithium have been done by replacing in the media sodium by lithium at experimental concentrations of 50 to 150 mM (3). For this reason, much of the research on lithium is irrelevant because these concentrations are in the toxic range. In chronic use the same results are often achieved with much lower concentrations than necessary in vitro. In therapeutic use lithium is administered orally as Li_2CO_3 after which it is absorbed from the gastrointestinal tract and distributed to the various tissues, such as heart, kidneys, muscle, bone and brain (7). The clearance of lithium is effected by glomerular excretion and tubular reabsorption in the same manner as sodium. In contrast with sodium, potassium and calcium, no great differences exist between intracellular and extracellular lithium concentrations in many tissues, in which aspect lithium behaves like magnesium. Higher concentrations are reported in bone (8), thyroid and probably also in kidneys (9).

2.3 BIOCHEMISTRY OF LITHIUM

The basis of many biological effects of lithium is a substitution for other cations, either Na/K or Ca/Mg. This substitution can have direct effect on transport and concentration of the substituted cations. Lithium can also replace the cations functionally, especially calcium and magnesium. This can lead to more indirect effects, e.g. the interference of lithium with microenvironment and structure of membranes and proteins. This interference can result in effects of enzymes

and regulation mechanisms of cells via modification of the response to messengers, such as cyclic nucleotides and biogenic amines. The following review of the literature contains inevitably simplifications with regard to some topics and the in vivo situation is undoubtedly far more complex.

2.3.1. Direct Replacement Effects of Lithium

Lithium can replace Na/K or Ca/Mg in transportsystems and so be transported through membranes. The most important enzymes in this respect are the Na, K - dependent - ATP ases (10). Lithium can substitute for both ions.
Lithium stimulates the Na, K-ATP ases of erythrocytes (11-14), of muscle cells (15, 16) and of brain cells (17). In some tissues no effect has been observed (18). Recently the transport processes of lithium in erythrocytes have been clarified by Duhm et al. (19).
It appeared that three processes are involved:

1. An active transport mediated by the Na,K-ATP ase (13,19).
2. A counter exchange process between Li and Na across the membrane (20-22).
3. A passive leakage into or out of the cell (19,23,24).

Other cations may influence lithium transport as was shown in cerebral cortex (25) and in erythrocytes (26).
Lithium interferes not only with Na,K transport but also with Ca,Mg transport processes. No conclusive picture has been presented on this topic, partly because of species specifity of the effects and also because of doubtful experimental conditions, such as the presence of EDTA in the media (10). The effects of lithium on two enzymes involved in these Ca/Mg transport, the Mg-dependent ATP ase and the Ca, Mg-dependent ATP ase, have been studied extensively (10,12,14,27-29). The results are conflicting but there is no doubt that lithium interferes with calcium exchange processes (30-32).
The interference of lithium with transport processes and the substitution of other cations induces changes in the electrolyte content of tissues and body liquids (33). For instance, the calcium and magnesium content of the blood is raised in

man (34).
Lithium can also interfere with other processes which require cations. Especially substitution of calcium has many important implications for the stimulus-secretion coupling and the release of neurotransmitters. Some reports contain indications that lithium can either replace calcium functionally or raise the intracellular calcium concentration for instance in neurones and cerebral tissue (35-40). Lithium probably liberates intracellular calcium in these tissues (41, 42) but a total substitution of calcium, also functionally, cannot be ruled out (43).

2.3.2. Indirect Replacement Effects of Lithium

Besides the cation replacement effects lithium also influences a number of enzymes by altering the spatial structure or environment of enzymes. The kinetics of enzymes that require magnesium are especially influenced by lithium (2). For this reason Birch suggested that the main mechanism of action of lithium is the interference with magnesium and/or calcium dependent processes (44). Important enzymes affected by lithium are those which are involved in the regulation of cellular functioning via the metabolism of the so called "messengers" e.g.:

- the biogenic amines, important for neurotransmission and brainfunction
- cyclic AMP, which plays a role in functionality of almost every cell.

Through these messengers and through modulation of various enzymes lithium affects various metabolic processes, such as carbohydrate metabolism (45), the metabolisms of amines and aminoacids (46,47) and DNA, RNA and protein synthesis (10).

Biogenic Amines

It is supposed that a number of mental disorders are caused by a disequilibration in the concentration of biogenic amines like serotonin, indolamine and dopamine. The concentration of

biogenic amines would be too high (in mania) or too low (in depression) (2). Conflicting results are reported on the effects of lithium upon the amines since it is reported that lithium has a therapeutic effect in both mania and depression (46). This would imply a sort of normalizing influence of lithium on the underlying biochemical mechanisms.

Cyclic AMP

Lithium inhibits in many cells the adenylate cyclase activity and lowers the cAMP concentration (48) as was demonstrated in brain cells (49-53),thyroid cells (9,54-56) and renal cells (57-60). In general no effect is observed on the activity of phosphodiesterases. The carbohydrate metabolism is affected by lithium via the cAMP system (45) partly through interference with the regulation of enzymes (61-63),partly through modulation of the response of adenylate cyclases on hormones such as glucagon (45). Not all the actions of lithium can be explained by the inhibition of adenylate cyclases and it has been suggested that lithium might also act on a site not related to adenylate cyclase (9, 69-73).
Still a few other adenylate cyclases are inhibited by lithium: the ACTH and fluoride stimulated adenylate cyclase of fat cells (64), the epinephrin and inositol stimulated enzyme of guinea pig heart (65) and the ACTH stimulated adenylate cyclase of rat adrenals (66). Lithium also affects the prostaglandin E_1 stimulated adenylate cyclase of platelets (67,68).

Concluding remarks

The actions of lithium clearly result from its similarity with other cations, especially magnesium. A number of cAMP mediated processes are affected by lithium induced inhibition of adenylate cyclase. Not all effects can be explained and other mechanisms (influence on protein kinase, Ca/Mg replacement or others) might be involved.

2.4. LITHIUM AND PLATELETS

The research on the effects of lithium on blood platelets

until now is mainly a reflection of the observed effects on other cells. The analogies between platelets and neurological cells have been the impetus for most investigations and much less the characteristic effects on platelets themselves.
Analogous to the research about the effect of lithium on the metabolism of biogenic amines in synaptosomes,the uptake of monoamines in platelets of lithium treated patients was investigated and turned out to be higher than in controls (69,70). Lithium did not exhibit these effects in human platelets in vitro and in rat platelets neither in vitro nor in vivo (71). Lithium stimulates in patients on lithium therapy the conversion of the monoamines in platelets via monoamine axidase (72,73).
There are only a few reports concerning the effect of lithium on platelet function. Geerdink et al. (74,75) showed that lithium stimulates the aggregation of human platelets after 90 min of incubation and especially prolonges the desaggregation phase. The stimulation of aggregation by lithium was observed after induction with ADP, adrenalin, collagen, serotonin and thrombin, as well in platelet-rich plasma as in gelfiltered platelets. Lithium counteracted the inhibitory effects of db-cAMP, PGE_1 and theophyllin. Also the impaired aggregation of thrombopathic platelets (uremia, release thrombopathia and after aspirin ingestion) was partly restored by lithium. Addition of lithium without further incubation inhibits the aggregation (75,76).
Lithium stimulates the lactate production of the glycolytic pathway and the incorporation of ^{14}C-glucose into glycogen, without any effect on nucleotide concentrations (77).
A possible interaction of lithium with calcium was deduced from the similarity of the effects of EDTA and lithium (77) and from the reducing of the instantaneous inhibitory effect of lithium by calcium (78). Anderson and Foulks (79) came to the same conclusion on the inhibitory effect of lithium on the aggregation of rabbit platelets. They suggested a competitive inhibition between lithium and calcium as mechanism for the action of lithium on platelets.
Lithium affects the platelet cyclic AMP-system: The PGE_1 sti-

mulated adenylate cyclase is inhibited by lithium in vivo and in vitro (after 45 min of incubation) (67,68). Lithium has no effect on the basal conversion of labeled ATP into cAMP. Magnesium enhances the stimulation of adenylate cyclase by PGE_1 and reduces the effect of lithium, which points to an identical binding site for lithium and magnesium on the enzyme. However, no relation with physiological function of platelets was made in either one of these publications.

A SHORT INTRODUCTION TO THE RESEARCH ON THE ACTION OF LITHIUM ON PLATELET BIOCHEMISTRY

From the literature about platelet biochemistry and the biological effects of lithium on cellular function as reviewed in chapter 1 and 2 the following mechanisms for the action of lithium were considered worthwhile for further research:

1. stimulation of platelet release reaction,
2. increase of cytoplasmatic calciumconcentration,
3. inhibition of platelet adenylate cyclase,
4. stimulation of platelet prostaglandin biosynthesis.

These aspects have been investigated one by one and the results of the investigations are described in the following chapters.

Chapter 3. An improved method for preparing platelet-rich plasma.
Thrombosis Research 11, 429-432, 1977.

For a variety of experiments it was necessary to use platelet-rich plasma, which was devoid of leukocytes and erythrocytes. An improved method for preparing platelet-rich plasma was developed, based on gradient centrifugation on a cushion of Ficoll-Isopaque. This method was used in most experiments except in those in which total cAMP had to be determined because the Ficoll-Isopaque appeared to interfere with the binding assay.

Chapter 4. The effect of lithium on platelet aggregation and platelet release reaction
Thrombosis Research 11, 297-308, 1977.

The uptake of lithium by platelets was measured in time and with different lithium concentrations. The dose-response effects and the influence of different incubation times of lithium on aggregation were investigated in relation to the intraplatelet lithium concentration. A pure stimulation of the release reaction as a possible explanation for the lithium effect, was excluded by the fact that lithium still enhanced the platelet aggregation when no release was measurable.

Chapter 5. Observations on ADP aggregation of lithium chloride incubated platelets in a variety of mammalian species.
Haemostasis 9, 276-287, 1980.

The experiments on rabbit platelets as reported by Anderson and Foulks (79), by whom an inhibitory effect of lithium on aggregation was shown, were extended by investigations on the effects of lithium on platelets of eight mammalian species. Lithium induces species-specific effects on the aggregation, ranging from stimulation to inhibition.
The effects of extracellular calcium do not support the suggestion of a competitive inhibition between lithium and calcium. A direct interference with the intraplatelet calcium is not evident from the effects of lithium on total intraplatelet calcium and magnesium and on ionophore A23187 induced aggregation.

Chapter 6. Lithium inhibits adenylate cyclase of human platelets.
Submitted for publication to Thrombosis and Haemostasis

The effect of lithium on the cAMP metabolism of human and rabbit platelets was investigated. Lithium does not induce changes in total cAMP content of human platelets, neither during incubation with lithium only , nor during subsequent

ADP induced aggregation. However, the adenylate cyclase activity of human platelets in rest and during aggregation is inhibited by lithium, especially after previous stimulation of the enzyme by prostacyclin. In rabbit platelets this lithium effect is not observed. The aggregation inhibiting effect of prostacyclin is counteracted by lithium in human platelets but not in rabbit platelets. This correlates well with the presence of a lithium effect on adenylate cyclase in human platelets and the absence of that effect in rabbit platelets.

Chapter 7. Lithium stimulates thromboxane B_2 formation in human platelets.
Submitted for publication to Prostaglandins

Since it is known that cAMP levels influence the platelet prostaglandin biosynthesis, the effect of lithium on PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 formation was measured.
Lithium stimulates the thromboxane B_2 synthesis in human platelets when aggregation is induced with agents, which at least partially induce aggregation by endoperoxide thromboxane formation. In resting platelets or when aggregation is induced with low concentrations of ADP no effect is observed. The synthesis of PGE_2 and $PGF_{2\alpha}$ is equally increased during aggregation of lithium incubated platelets compared to controls. In rabbit platelets no stimulation of lithium on thromboxane B_2, PGE_2 and $PGF_{2\alpha}$ formation is observed.
In conclusion: the mechanism of the aggregation promoting effect of lithium in human platelets is most likely an inhibition of adenylate cyclase, by which the platelet thromboxane synthesis is increased during aggregation induction with proper agents.

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CHAPTER 3

AN IMPROVED METHOD FOR PREPARING PLATELET-RICH PLASMA

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INTRODUCTION

The contaminating leucocytes and erythrocytes can interfere with determinations of platelet metabolic activity and functionality in platelet-rich plasma. For experiments concerning the effect of lithium on human platelets (1) an improved method was developed for the preparation of platelet-rich plasma, poor in leucocytes and erythrocytes and assuring high recovery of platelets.

MATERIAL AND METHODS

Preparation of Platelet-Rich Plasma

Blood from healthy volunteers was collected in polypropylene tubes and anticoagulated with 0.1 volume of 3.8 % trisodium citrate dihydrate (w/v). Platelet-Rich Plasma (PRP) was prepared as usual by centrifugation of the blood (10 min, 250 G at room temperature) or by centrifugation on a Ficoll-Isopaque cushion (Ficoll: MW 4 x 10^5, Sigma; Isopaque: Nyegaard) prepared according to Loos and Roos (2).
Two parts of blood were carefully layered with a polypropylene pipette on one part of Ficoll-Isopaque (density 1.070 g/ml at 25°C, containing 0.38 % trisodium citrate dihydrate and a trace of dextran blue (MW 2 x 10^6, Sigma). After centrifugation for 20 min at 250 G at room temperature, PRP was pressed out of the tube by pumping a sucrose solution with a density of 1.075 g/ml into the Ficoll-Isopaque layer. PRP was stored in capped polypropylene tubes at room temperature and used within 120 minutes. The platelet count in PRP samples used for aggregation experiments was adjusted at 10^8/ml with platelet-poor plasma prepared by centrifugation of citrated blood at 4000 G for 10 min.

Aggregation

Platelet aggregation was measured photometrically (3) with a Vitatron UC 200 colorimeter (at 600 nm) equipped with a

Kipp-recorder. Cuvettes were thermostated at 37°C and contained 2 ml PRP that was stirred at 1100 rpm with a plastified stirring bar. The maximal change in extinction was expressed in units.

Metabolic parameters

The content of ATP, ADP and AMP in platelets and the lactate production were determined enzymatically (4). Oxygen consumption of platelets was determined at 37°C in a closed thermostated cuvette with a capacity of 4 ml, and equipped with a stirring bar and a Clarke-type pO_2-electrode (Radiometer, E5046).

RESULTS

Quality control of PRP prepared by two different methods

PRP was isolated from the same blood samples, by differential centrifugation (normal-PRP) as well as by centrifugation on a Ficoll-Isopaque cushion (Ficoll-PRP). Some functional and metabolic properties of these PRP samples were compared and the data are summarized in Table I.
The recovery of platelets in Ficoll-PRP was better and contamination with erythrocytes and leucocytes was smaller. When Ficoll-PRP was isolated very carefully, recovery of platelets amounted 80-96 %.
Ficoll-Isopaque itself did not cause changes in platelet functionality. The aggregation response representing the functionality, was sometimes even better in Ficoll-PRP. Also the release properties of the platelets could still be found after Ficoll-PRP preparation, as can be seen from results published elsewhere (1).
These results indicate that no harm was done to functional properties of the platelets. To get an idea of the biochemical properties of the platelets after Ficoll-PRP preparation, a few metabolic parameters were also determined. As Table I suggests no significant differences in metabolic activity exist.

TABLE I

Comparison of normal PRP with Ficoll-PRP

Parameter		Normal PRP	Ficoll-PRP	
Recovery of platelets (n = 15)		60 ± 10	88 ± 8 percent	
Contaminating Erythrocytes/10^5 platelets (n = 6)		40 - 200	4 - 20	
Contaminating leucocytes/10^5 platelets (n = 6)		10 - 100	< 4	
Aggregation* (n = 8)		100	100 - 125 units	
ATP (n = 6)		4.3 ± 0,2	4.3 ± 0.2	μmol/10^{11} pl.
ADP (n = 4)		3.6 ± 0.3	3.8 ± 0.6	μmol/10^{11} pl.
AMP (n = 2)		0.44± 0.04	0.50± 0.08	μmol/10^{11} pl.
O_2 consumption (n = 4)		52 ± 6	56 ± 8	μmol/10^{11} pl/hr
Lactate production	Exp. 1	128	117	μmol/10^{11} pl/hr
	Exp. 2	214	230	μmol/10^{11} pl/hr

*Aggregation of normal PRP taken as 100 units (2.1 x 10^{-6}m ADP)

Data: mean ± S.D.

DISCUSSION

Since erythrocytes and leucocytes may affect platelet metabolism and aggregation (5,6), admixture of these blood cells has to be minimal; and, in order to prevent selective loss, maximal recovery of platelets has to be ensured. Moreover, it is preferable to keep the platelets in their natural environment (7). The usual platelet isolation methods, e.g. differential centrifugation and gelfiltration, do not fulfill these requirements (6). The preparation of platelet-rich plasma on a Ficoll-Isopa-

que cushion, as described in this paper, largely overcomes these problems. Recovery of platelets amounts to about 90 %. Neutral polymers like Ficoll have no effect on platelet adhesiveniss, ADP-induced aggregation and release in vitro (8). These findings were confirmed in this study and extended to some metabolic parameters.

The aggregability of platelets prepared by this method at least equals that of platelets isolated by differential centrifugation, suggesting that no harm is done to platelet populations by this isolation method.

Ficoll-PRP has been used extremely satisfactory throughout all experiments.

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CHAPTER 4

THE EFFECT OF LITHIUM ON PLATELET AGGREGATION AND PLATELET RELEASE REACTION

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ABSTRACT

Lithium increases in vivo and in vitro the aggregating potency of human platelets. One of the mechanisms presumed to underlie this aggregation-promoting effect, potentiation of ADP release, was investigated in relation to lithium uptake. It appeared that lithium is not taken up by human platelets against a concentration gradient, and equilibrium is achieved in about 120 min. When platelets are incubated for this fixed time with different lithium concentrations, the aggregation response is related to the intraplatelet lithium concentration. When platelets are incubated during increasing periods with a fixed lithium concentration, no direct correlation exists between intraplatelet lithium concentration and extent of aggregation. After blocking the release with aspirin, the stimulatory effect of lithium on platelet aggregation was still observed.
Low ADP concentrations, causing no measurable release, still caused more marked aggregation of platelets preincubated with lithium chloride than of platelets incubated with sodium chloride. It is concluded that the increase in platelet aggregation which occurs after incubation of platelets with lithium, is not caused merely by potentiation of the release reaction.

INTRODUCTION

Lithium increases the intensity of platelet aggregation and prolongs the disaggregation phase (1). This increase is already observed at concentrations of 1 mmolar as attained in the blood during oral lithium treatment of patients with mental diseases (2,3,4). The promoting effect on the aggregation phenomenon is independent of the kind of stimulus which induces the aggregation. An essential prerequisite for potentiation of in vitro aggregation is preincubation of the platelets with lithium (1,5). Without incubation, lithium even reduces the intensity of aggregation (5). The mechanism by which lithium influences cell behaviour is not known. Because of the therapeutic effect of lithium in mental diseases, changes in neuronal synapse transfer mechanisms and in other target cell membrane functions have been suggested (6,7).

In this study the hypothesis was tested whether lithium can change the platelet aggregability by influencing the platelet release reaction. The platelet release reaction was studied by measurement of ^{14}C-serotonin release (8) and related to platelet aggregation pattern and intraplatelet lithium concentration.

It is shown that the effect of lithium on platelet aggregation cannot be explained by potentiation of the platelet release reaction only.

MATERIAL AND METHODS

Preparation of platelet-rich plasma

Blood from healthy volunteers was collected in polypropylene tubes and anticoagulated with 0.1 volume of 3.8 % trisodium citrate dihydrate (w/v). Platelet-Rich Plasma (PRP) was prepared as described elsewhere (9) by centrifugation on a Ficoll-Isopaque cushion. The platelet count in PRP samples used for aggregation and release experiments was adjusted at 10^8/ml with platelet-poor plasma prepared by centrifugation of citrated blood at 4000 G for 10 min.

Aggregation

Platelet aggregation was measured photometrically (10) with a Vitatron UC200 colorimeter (at 600 nm) equipped with a Kipp recorder. Cuvettes were thermostated at 37°C and contained 2 ml PRP that was stirred at 1100 rpm with a plastified stirring bar.
To compensate for intra-individual differences in aggregability between PRP samples, the following procedure was used: from each PRP, two ml samples were incubated in duplicate with 10 mM lithium for 120 minutes. The maximal change in optical density during aggregation of these standard samples with a certain concentration of ADP, was defined as 100 units of aggregation. By comparison with this change in optical density, the aggregatory responses of other samples of that PRP specimen could be expressed in units.

Determination of lithium concentration in platelets

Samples of platelet-rich plasma were incubated during various periods with varying lithium chloride concentrations at 37°C. After incubation platelets were separated from plasma with the aid of modified cytocrit tubes (11). Tubes with a length of 63 mm and an internal diameter of 7 mm were fused to haematocrit capillaries with a length of 75 mm and an internal diameter of 0.4 mm. The capillary end was sealed and the capillary part was filled with Ficoll-Isopaque (density 1.045 g/ml and containing 0.38 % trisodium citrate dihydrate). 0.2 ml of the same Ficoll-Isopaque, to which 1 mg EDTA/ml had been added, was pipetted into the tubes. EDTA was added to this Ficoll-Isopaque solution to prevent clumping of the platelets in the shoulder of the tubes during sedimentation.
On the Ficoll-Isopaque cushion, 2 ml PRP was carefully layered and the platelets were separated from the plasma by centrifugation at 4000 G during 10 min. In control experiments with platelets preloaded with serotonin, it was observed that this

platelet isolation method did not cause any release. In the plasma layer that was sucked off, the platelet count was determined. The capillaries containing the sedimented platelets were frozen immediately in dry-ice/acetone. The capillaries were cut off just above the platelet column and the packed platelets were resuspended in 3 ml double distilled water. After lysis by freezing and thawing the platelet fragments were sedimented by centrifugation at 4000 G during 10 min. The lithium concentration in the supernatant was determined with an Eppendorf emission flame photometer. In the experiments with a plasma lithium concentration of 1 mM it appeared to be necessary to dissolve the platelets from at least 3 cytocrit tubes in 3 ml water. To exclude the influence of other ions in these lithium determinations (12), a blank of lysed platelets not incubated with lithium was used. In parallel experiments, ^{3}H-Inuline (750 mCi/mmole; 0.5 μCi/ml PRP)was added before centrifugation and the amount of trapped plasma was calculated from the radioactivity in the platelet sediment. The lithium concentration is expressed in nanomoles per 10^{8} platelets. Since it is shown that the fluid volume of 10^{8} platelets amounts to 1 μl (13,14) the concentration in nanomoles/10^{8} platelets corresponds with mmoles/l cell water.

Serotonin uptake

Samples of 2 ml PRP were incubated for 60 and 120 minutes at 37°C with identical concentrations of LiCl and NaCl. 5 μl ^{14}C-serotonin (55 mCi/mmole, 5 μCi/ml, Amersham) was then added and, at appropriate time intervals, 100 μl samples were taken and diluted in 0.5 ml ice-cold saline containing 0.4 % w/v EDTA (15). After centrifugation at 4°C (4000 G, 5 min) 400 μl samples of the supernatant were added to 9 ml Instagel (Packard) and radioactivity was determined with a Liquid Scintillation Counter (LKB 81000). The percentage of radioactive serotonin taken up by the platelets was calculated from the radioactivity added to PRP.

Serotonin release

PRP samples of 2.5 ml were incubated for 120 min at 37°C with identical concentrations of LiCl or NaCl and with 10 µl ^{14}C-serotonin. Then aggregation was induced by addition of ADP and the ^{14}C-serotonin appearing in the plasma determined in the way as described above. The amount of ^{14}C-serotonin released from the pre-loaded platelets into the plasma, was expressed as percentage of the initial ^{14}C-serotonin content.

RESULTS

Uptake of lithium ions

Preliminary experiments showed that lithium ions do not attach to the outside of human platelets, for no lithium was found in platelet pellets isolated immediately after addition of lithium chloride to PRP. The lithiumcontent in platelets increased with

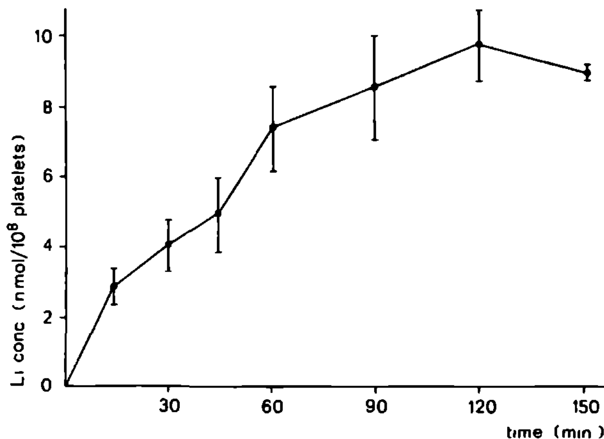


FIGURE 1

Lithium concentration in platelets during incubation with 10 mM LiCl (mean ± S.D., n = 6).

time of incubation and a plateau level was reached in about 120 minutes (Fig. 1). From this figure it can be concluded also that at the plateau level the intraplatelet lithiumconcentration equals that of the surrounding plasma. The lithium content of platelets after 120 minutes of incubation with different concentrations of lithium chloride is presented in Table I and appears to correlate fairly well with the plasma concentrations.

TABLE I

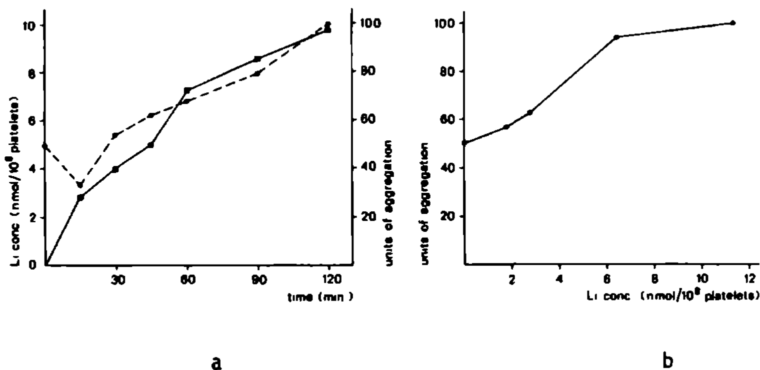
Concentration of Lithium in Blood Platelets

1 mM LiCl:	1.33 ± 0.36	(n = 9)
5 mM LiCl:	5.16 ± 1.38	(n = 10)
10 mM LiCl:	10.56 ± 1.80	(n = 17)

Concentrations in nanomoles/10^8 platelets after 120 min incubation at 37°C, with the indicated plasma lithium concentration (mean ± S.D.).

Relation between intraplatelet lithium concentration and potentiation of aggregation

To investigate the extent to which the potentiating effect of lithium on platelet aggregation depends on the intraplatelet lithium concentration, aggregation was induced with low ADP concentrations (1-2 x 10^{-6}M), which did not induce a secondary release. Aggregation was induced in all PRP samples after storage for 120 min at 37°C. At different time intervals before aggregation induction, 10 mM lithium chloride was added in order to achieve different incubation times. In control experiments, sodium chloride instead of lithium chloride was added in order to exclude a possible influence of hypertonicity (16). Addition of 10 mM NaCl appeared to have no effect on platelet aggregation, neither immediately nor in time.



a b

FIGURE 2

Figure 2a: Effect of platelet incubation with 10 mM LiCl on ADP-induced aggregation (----) and intraplatelet lithium concentration (——) during Aggregation was induced with 1.6 x 10^{-6} M ADP.

Figure 2b. Effect of different intraplatelet lithium concentrations after 120 min incubation with the appropriate LiCl concentrations on ADP induced aggregation. Aggregation was induced with 1.6 x 10^{-6} M ADP.

No aggregation: 0 units aggregation; max. aggregation with LiCl: 100 units aggregation.

In Fig. 2a intensity of aggregation and intraplatelet lithium concentration are plotted against incubation time (means of 3 experiments). After 15 min incubation with 10 mM of LiCl, about 3 nmoles lithium/10^8 platelets had been taken up, at which time the aggregation intensity was lower than in the control. This lowering of the aggregation intensity after short incubation with 10 mM LiCl was observed in each experiment. Addition of 10 mM sodium or rubidium chloride did not reduce the aggregation intensity after the same period of incubation. After 30 min incubation about 4 nmoles lithium/10^8 platelets had been taken up, at which time the intensity of aggregation was almost equal to that in the control. After longer incubation, the aggregation intensity increased considerably.
In another type of experiment aggregation was induced in PRP samples incubated during 120 min with different concentrations of lithium chloride (Fig. 2b). After this time of incubation

equilibrium is reached and even an intraplatelet lithium concentration as low as 2 nmoles/10^8 platelets caused an increase in aggregation intensity which was not observed after incubation with the same concentration of sodium chloride.

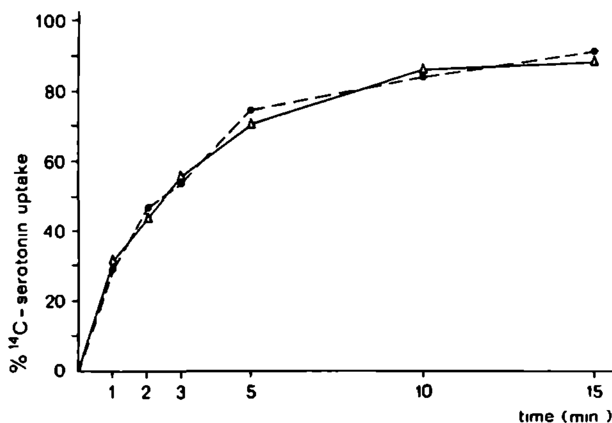


FIGURE 3

Uptake of ^{14}C-serotonin in blood platelets at 37°C after incubation with 10 mM NaCl (----) or LiCl (——)

Uptake of ^{14}C-serotonin

The uptake of ^{14}C-serotonin by platelets after preincubation with either 10 mM LiCl or NaCl was examined to exclude a possible effect of lithium since reports concerning this process are contradictory (17,18). As Fig. 3 shows lithium exerts no influence on the quantity or velocity of serotonin uptake.

Effect of lithium on primary aggregation

From the preceding experiments it is concluded that preincubation of platelets with lithium influences aggregability. After a short incubation period lithium reduces aggregation intensity, whereas after longer incubation periods aggregation is intensified, which

might be due to potentiation of the platelet release reaction. In order to examine this release phenomenon, aggregability and ^{14}C-serotonin release of platelets incubated with 10 mM NaCl or LiCl were studied after blocking the release reaction by aspirin. It appeared that after aspirin ingestion, no serotonin release was measurable.

TABLE II

Effect of Lithium on Aspirin-treated Platelets

	Preincubation with 10 mM	^{14}C-serotonin release (%)	Units of aggregation
Before aspirin	NaCl	3 $\pm$ 1	39 $\pm$ 12
	LiCl	55 $\pm$ 7	100*
After aspirin	NaCl	0	34 $\pm$ 12
	LiCl	0	70 $\pm$ 15

Aggregation and release of ^{14}C-serotonin of PRP incubated with 10 mM NaCl or LiCl (120 min, 37°C), before and after aspirin ingestion (1000 mg ASA, 2 hrs before venepuncture). ADP concentration: 4.2 x 10^{-6}M. All data are the mean $\pm$ S.D. of four experiments.

*Defined as 100 units of aggregation.

Nevertheless the lithium incubated platelets still showed an increased aggregation compared to the control platelets incubated with sodium chloride (Table II).

In an other type of experiments, aggregation in PRP was induced with decreasing quantities of ADP until no serotonin release was measurable. Even at these low ADP concentrations, lithiumincubated platelets showed more pronounced aggregation (Table III).

^{14}C-serotonin release during ADP-induced aggregation

The effect of lithium on primary aggregation shows that the aggregation promoting effect is obviously not due to release potentiation. However, strong aggregation may by itself induce a secondary release. This aspect was investigated with ADP concentrations as used in the preceding sections ($2\text{-}4 \times 10^{-6}$M). Representative aggregation- and release patterns of PRP incubated with 10 mM NaCl or LiCl, are shown in Fig. 4.

TABLE III

Effect of Lithium on Primary Aggregation

ADP conc. $\times 10^{-6}$M	preincubation with 10 mM	units of aggregation		^{14}C-serotonin release (%)	
4.2	NaCl	53	45	3	1
	LiCl	100	100	59	35
2.1	NaCl	37	33	0	0
	LiCl	64	54	6	3
1.0	NaCl	n.d.	20	n.d.	0
	LiCl	n.d.	29	n.d.	0
0.5	NaCl	1	3	0	0
	LiCl	12	14	0	0

Aggregation and ^{14}C-serotonin release induced by different ADP concentrations after incubation during 120 min at 37°C with 10 mM NaCl or LiCl (two experiments).

The aggregation of NaCl-incubated PRP was reversible and no release was observed. After incubation with LiCl the sensitivity of the platelets to ADP was increased to such an extent that aggregation was irreversible with a concomitant serotonin release of 80 %. The observation that after 1 min of aggregation

a great difference in aggregation intensity occurs before release is observed in either sample, suggests that the release in lithium-incubated PRP results from the intensified aggregation.

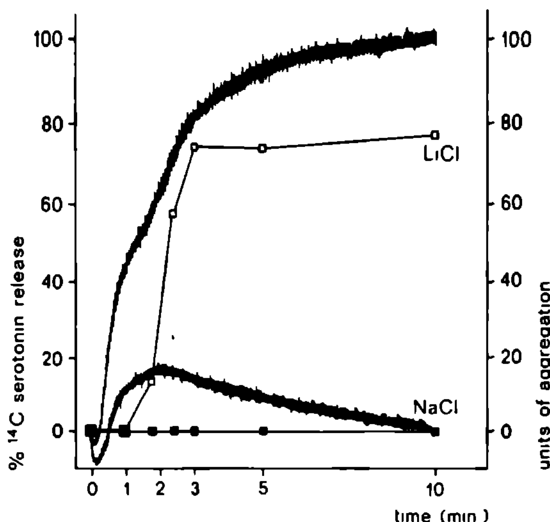


FIGURE 4

Time curve of ADP-induced aggregation and release after 120 min incubation with 10 mM LiCl or NaCl. ADP concentration: 4.2×10^{-6}M.

DISCUSSION

Lithium increases as well in vivo as in vitro the aggregating potency of platelets. A prerequisite for this action of lithium is preincubation of the platelets with this ion. After preincubation, even concentrations of 1 and 2 mmolar have a stimulatory effect on the aggregability. Without preincubation higher concentrations reduce the aggregation intensity (5). For this reason intraplatelet lithium concentration was studied in relation to the aggregability of the platelets.

The platelet aggregation-promoting effect of lithium depends on concentration and incubation time. This lithium effect is not due to changes in osmolarity (16) or membrane potential since

the effects were not observed after incubation with the same amounts of NaCl or RbCl. It appears that the time of preincubation is more important than the actual intraplatelet lithium concentration attained. After short incubation, lithium reduces the aggregation intensity even when the intraplatelet concentration already amounts to 3 mmolar. The mechanism of this aggregation-reducing effect remains unsolved, but it is possibly an effect on the outside of the platelet membrane.

More important are the effects after long-term incubation of platelets with lithium, since these resemble the in-vivo situation. After 90-120 min incubation at 37°C, the intraplatelet lithium concentration equals the plasma concentration. Such an equilibrium has also been observed in muscle cells (19). At equilibrium the aggregation response correlates well with intraplatelet lithium concentration (Fig. 2b) and even low concentrations of LiCl (1 mM) induce an increased aggregation response. These results suggest an action of lithium on biochemical processes in the platelet. One of the possible mechanisms is potentiation of the release of ADP, that runs parallel to the release of ^{14}C-serotonin (8).

Our investigations do not support this hypothesis. If lithium acted solely on the release reaction, then no differences in primary aggregation, induced by low concentrations of ADP, would be observed between lithiumincubated samples and controls. But lithiumincubated samples still aggregate better than controls. Also after blocking the release completely with aspirin lithium preincubation enhances the aggregation. From these observations it can be concluded that the increase of aggregation is not only caused by potentiation of the release reaction. So, other possible mechanisms of this lithium effect have to be considered like an inhibition of adenylcyclase (20),a replacement of intracellular calcium or magnesium (19),or changes in platelet prostaglandin- and thromboxane synthesis (21). These aspects are at the moment under study.

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CHAPTER 5

OBSERVATIONS ON ADP-AGGREGATION OF LITHIUM-CHLORIDE INCUBATED PLATELETS IN A VARIETY OF MAMMALIAN SPECIES

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ABSTRACT

In citrated platelet-rich plasma of eight mammalian species lithium produces species dependent effects on ADP-aggregation ranging from stimulation to inhibition. Raising the extra-cellular calcium concentration enhanced aggregation of both lithium-stimulated and lithium-inhibited platelets, indicating that the extracellular calcium concentration has no direct relation to the effect of lithium on platelet function. Lithium did not induce changes in the total intraplatelet calcium and magnesium contents. Aggregation of platelets with ionophore A23187 showed the same species-specific lithium effects as aggregation induced by ADP. The data suggest that lithium does not exert its effects on platelets through interference with intra- or extracellular calcium concentrations.

INTRODUCTION

Addition of lithium to platelet-rich plasma has an instantaneous inhibitory effect on the ADP-induced aggregation (1, 7, 9). After preincubation with lithium this inhibitory effect is maintained in rabbit platelets (1), but the aggregation of human platelets appears to be stimulated considerably (6,9). This latter effect occurs even at very low lithiumconcentrations of about 1 mM (9) which are reached in patients under lithium therapy.
The mechanism of action of lithium on platelets is obscure.
We could show that lithium does not stimulate human platelet aggregation merely by potentiating the release reaction (9).
One of the proposed mechanisms of action in other biological systems is an interaction of lithium with calcium- and/or magnesium-dependent processes (2,13). Calcium plays an essential role in platelet aggregation not only extracellularly (4) but also intracellularly (12). Anderson and Foulks (1) reported that the inhibition of the ADP-induced aggregation of rabbit platelets by lithium is antagonized by increasing the calcium concentration of the citrated plasma in which they are suspended. In view of a possible relation between intracellular calcium and/or magnesium contents and the effect of lithium on platelets, we investigated the effect of lithium on the aggregation of a variety of mammalian platelets with different intracellular calcium and magnesium contents.

MATERIAL AND METHODS

Preparation of Platelet-Rich Plasma (PRP)

Human platelet-rich plasma was prepared as described previously (10) by centrifugation of blood, anticoagulated with 1/10 volume of 3.8 % (w/v) trisodium citrate, on a Ficoll-Isopaque cushion (density 1.070 g/ml) for 20 min at 250 G.
Animal blood, anticoagulated in the same manner with trisodium citrate, was obtained from Rhesus monkey, New Zealand white rabbit, Labrador dog, cat, sheep, cow and pig. Since preparation

of PRP by the Ficoll method resulted in insufficient separation of the erythrocytes from the plasma (e.g. in cow and rabbit), animal PRP was prepared by differential centrifugation (10 min at 300 G).
The platelet count of PRP was determined with a Coulter Counter model B at appropriate settings. For aggregation experiments, PRP was diluted with autologous platelet-poor plasma (prepared by centrifugation of blood at 4000 G for 10 min) to a final platelet count of 2-3 x 10^8/ml.

Aggregation of Platelets

Aggregation was measured essentially according to Born (3) with some minor modifications (9). PRP-samples of 1 ml were incubated for 90-120 min at 37°C in all experiments before inducing aggregation. Aggregation was induced either by ADP (Boehringer) dissolved in Veronal buffer or by ionophore A23187 (a gift from Eli Lilly Comp.). The ionophore was stored in DMSO at a concentration of 1 mg/ml. Just before use a sample was thawed and diluted to appropriate concentrations.
Ten µl samples of lithium chloride or sodium chloride (Merck) were added at appropriate time intervals before aggregation induction to establish a final concentration of 10 mM. In the experiments in which the plasma calcium concentration was changed, $CaCl_2$ (Merck) was added either 90 min before or just before aggregation induction. Samples incubated for the same time with NaCl were used as controls.

Measurement of Platelet Calcium and Magnesium Contents

In order to measure platelet calcium and magnesium content it is necessary to separate the platelets from the plasma. This was accomplished by layering 2 ml of PRP on 0.75 ml of a 10 % (w/v) solution of Ficoll 400.000 (Sigma) in isotonic choline chloride (Merck) (density 1.045 g/ml) in a polystyrene tube and centrifugating for 8 min at 4000 G in a swing-out rotor (Sorvall HB_4) at room temperature.

The platelet poor plasma was pipetted off and the tubes were frozen immediately in dry-ice/methanol. The platelet pellet was cut off and resuspended in 3 ml of a 5 % (w/v) solution of trichloroacetic acid (Merck) with 0.1 % (w/v) La_2O_3 (Merck) and left overnight at 4^oC. Then the flocculated proteins were spun down and the amount of calcium and magnesium in the supernatant was determined with a Perkin-Elmer Atomic Absorption spectrophotometer (model 403). The amount of trapped plasma was measured in parallel experiments with ^3H-Inulin (Amersham) using a LKB-Liquid Scintillation Counter 81000. In control experiments it was observed that this platelet isolation method did not cause any measurable serotonin release (9).

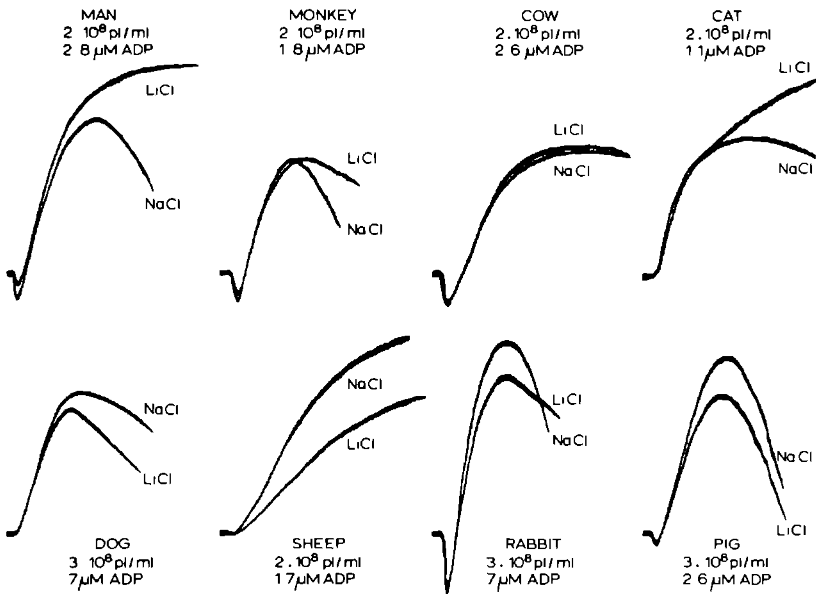

FIGURE 1

ADP-induced aggregation curves of mammalian platelets after 90 minutes of preincubation with 10 mM NaCl or LiCl.

Uptake of Lithium into Platelets

The amount of lithium taken up by the mammalian platelets after 90 min of incubation with 10 mM LiCl was measured as described previously (9). In short: after incubation with 10 mM LiCl the platelets were separated from the plasma by centrifugation through a Ficoll-Isopaque layer, and the amount of lithium in the pellet was determined with an Eppendorf emission flame photometer.

RESULTS

Effects of Lithium on ADP-induced Aggregation of Mammalian Platelets

The effect of lithium on the ADP-induced aggregation of platelets from different mammals after 90 min of incubation differs markedly. Typical examples of aggregation patterns are shown in Fig. 1. and were obtained in PRP samples from at least 5 specimens of each species. Not only the rate of aggregation, but also the maximal aggregation is influenced by lithium. The effect on maximal aggregation ranges from an increase (in man and cat) through almost no effect (cow and monkey) to a decrease (dog, rabbit, pig and sheep). In order to detect these effects of lithium on the aggregability, only submaximal ADP concentrations were used (Fig. 1). The tendency of the effect of lithium is uniform in each species but the actual amount of inhibition or stimulation varied interindividually to about 10 %.
The velocity of desaggregation was decreased in man, monkey, cat and rabbit, whereas the steepness of aggregation was only lowered in sheep.

Time- and ADP-concentration Dependency of the Lithium Effect on Aggregation

The effect of different incubation times with sodium and lithium from 0 min (aggregation-induction immediately after addition of NaCl or LiCl) up to 90 min, were investigated.

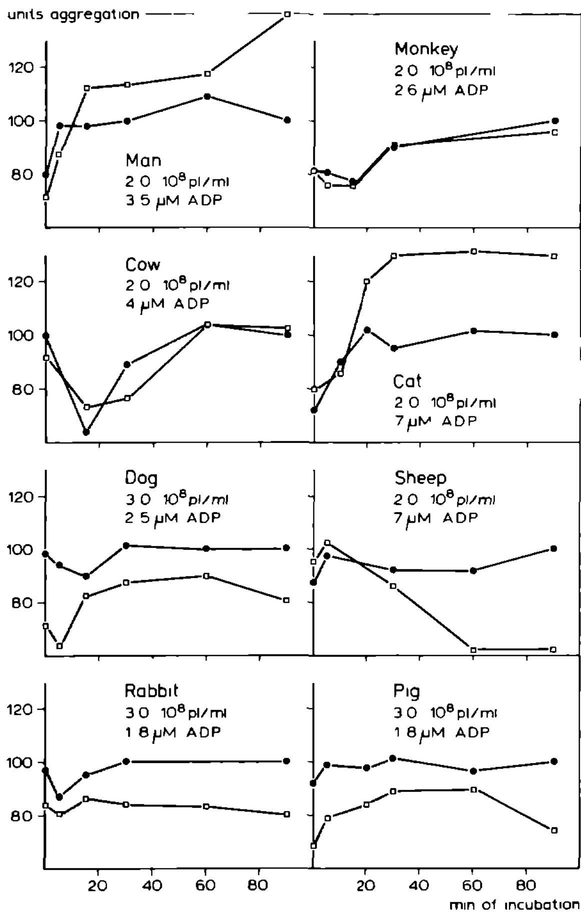

FIGURE 2

Time dependency of the effect of 10 mM NaCl or LiCl on the aggregation of mammalian platelets (●: NaCl; ◻: LiCl).
The points of zero min of preincubation were obtained by inducing aggregation immediately after adding NaCl or LiCl. The aggregation of the samples incubated with NaCl for 90 minutes, was taken as 100 units of aggregation for each species.

In almost all kinds of platelets a short incubation from 0 up to 10 min with sodium and lithium induces an inhibition of the aggregation (Fig. 2).
After prolonged incubation periods, however, a great variety in aggregation patterns is observed. In human and cat platelets from 30 min of incubation on, lithium produces a pronounced increase of aggregation. The intensity of aggregation (OD_{max}) of lithium incubated and control bovine-platelets returns to the starting values after 60 min of lithium incubation.
The decreased aggregation of platelets of dog and pig just after addition of sodium or lithium, improves after prolonged incubation both with lithium and sodium, although aggregation of lithium incubated samples remains impaired.

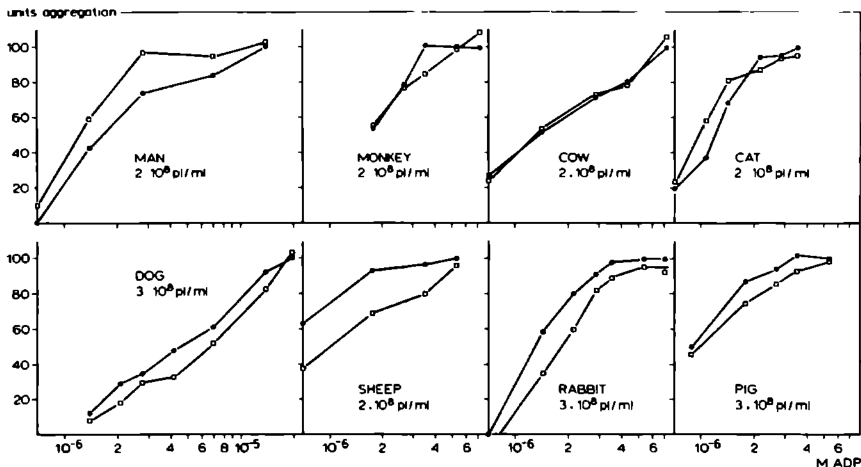

FIGURE 3

ADP dose-response curves for mammalian platelets after 90 min of preincubation with 10 mM NaCl or LiCl (●: NaCl; □: LiCl).
The maximal aggregation of NaCl incubated PRP was taken as 100 units of aggregation for each species.

In rabbit platelets the initial degree of lithium inhibition remains almost unchanged up to 90 min of preincubation.
In sheep platelets lithium produces a distinct inhibition of the aggregation only after prolonged incubation times.
The sensitiveness of platelets for ADP varies considerably between different species and individuals. This leads to spreading of the degree of stimulation or inhibition by lithium. To get a more precise picture of the effect of long-term lithium preincubation on aggregation, ADP dose-response curves were made of which Fig. 3 shows typical examples. The results again illustrate the variety in effects between the different species. In platelets from some dogs lithium produces an increased aggregation with high ADP concentrations (> 10^{-5}M ADP) but mostly the effect remains inhibitory.

Lithium Effect and Extracellular Calcium

The results above indicate that there are at least two different effects of lithium; an instantaneous inhibitory effect and a long-term effect, varying from inhibition to stimulation. Addition of calcium to PRP after lithium preincubation increases the aggregation in platelets which show decreased aggregation (rabbit), as well as in platelets which show an increase of aggregability (human). Table I shows that raising the calcium concentration increases the aggregation of both lithium-incubated samples and controls up to a maximum. This is observed when calcium was added 90 min before as well as just before aggregation induction.

Lithium Effect and Intracellular Calcium

Large differences exist in divalent cation contents in the platelets of different species (Table II). To study the interaction of lithium with calcium and magnesium,the content of these ions in various mammalian platelets was determined after incubation with either 10 mM NaCl or LiCl. Control experiments revealed that lithium was taken up into the platelets of the species

TABLE I

Effect of changing the extracellular calcium concentration on the ADP-induced aggregation of citrated PRP

		Human platelets		Rabbit platelets	
		10 mM NaCl	10 mM LiCl	10 mM NaCl	10 mM LiCl
no addition		100*	126	100*	74
3 mM $CaCl_2$	0 min.inc.	140	167	136	115
	90 min.inc.	167	167	130	120
5 mM $CaCl_2$	0 min.inc.	162	174	147	143
	90 min.inc.	158	170	spontaneous aggregation	

PRP was incubated for 90 min with 10 mM NaCl or LiCl.
$CaCl_2$ was added either at the beginning of the incubation period (90 min), or after 90 min of incubation just before aggregation induction (0 min).
Aggregation was induced with 2.4 µM ADP for human platelets and with 0.7 µM ADP rabbit platelets.

*The maximal aggregation of these samples was defined as 100 units of aggregation.

tested (man, cat, dog, sheep) till the intracellular concentration was about equal to the extracellular lithium concentration. This is in accordance with the results in other cells (13). Due to the uptake of lithium, 3-10 nanomoles of calcium and/or magnesium might be expelled from 10^8 platelets depending on their volumes. In fact, incubation with 10 mM LiCl did not induce a loss of that magnitude (Table II). No relation at all was found between the effect of lithium on aggregation of the platelets and calcium-(Fig. 4a) or magnesium content (Fig. 4b) (e.g. cat and pig thrombocytes have almost the same calcium and magnesium contents but show different lithiumeffects).

TABLE II

Total calcium and magnesium content of control and lithium-incubated platelets.

		Calcium	Magnesium	Number of experiments
MAN	Control	22.8 ± 4.0	8.8 ± 2.3	8
	Li	23.0 ± 4.4	8.8 ± 2.7	8
MONKEY	Control	28.4 ± 5.3	9.4 ± 1.4	6
	Li	30.4 ± 5.6	9.7 ± 1.1	3
COW	Control	14.8 ± 1.3	11.2 ± 2.0	4
	Li	13.5 ± 1.3	10.9 ± 2.6	2
CAT	Control	6.8 ± 1.1	24.9 ± 2.3	4
	Li	5.5 ± 0.2	25.9 ± 2.3	2
DOG	Control	7.0 ± 2.1	13.6 ± 3.0	8
	Li	6.1 ± 2.1	12.2 ± 2.1	4
SHEEP	Control	6.7 ± 1.7	8.0 ± 0.7	4
	Li	6.9 ± 2.2	8.0 ± 1.4	4
RABBIT	Control	10.3 ± 2.6	13.2 ± 0.9	4
	Li	10.8 ± 4.3	12.9 ± 0.3	4
PIG	Control	9.9 ± 2.3	27.4 ± 4.8	4
	Li	9.7 ± 3.9	28.2 ± 7.8	2

Platelets were incubated for 90 minutes with 10 mM NaCl (control) or LiCl.
Thereafter the calcium and magnesium content was determined and expressed in nanomols/10^8 platelets (mean ± S.D.)

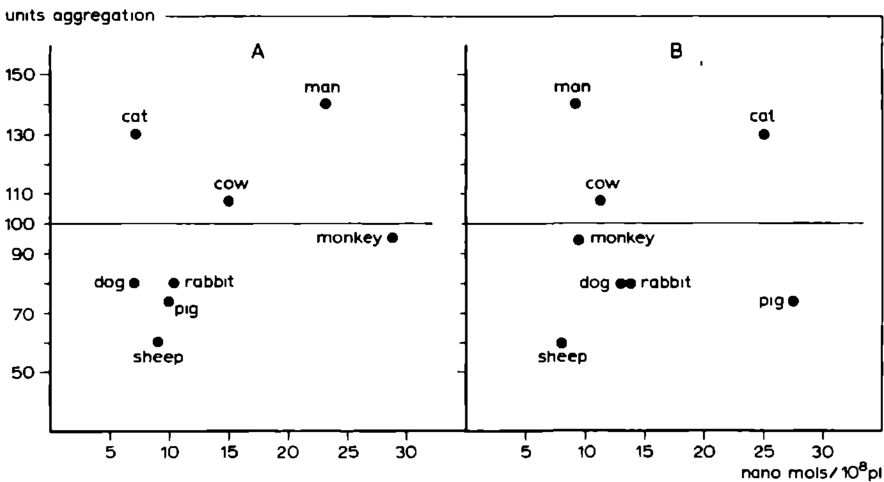

FIGURE 4

Calcium and magnesium content of mammalian platelets in relation to the effect of lithiumpreincubation on ADP induced aggregation.
PRP was incubated for 90 min with 10 mM LiCl prior to aggregation induction. Control aggregation of each species was taken as 100 units of aggregation.
A Calcium content versus lithium effect
B Magnesium content versus lithium effect.

Lithium could also interfere with the intracellular calcium shifts which are supposed to be essential for aggregation induction (12). Aggregation of platelets with ionophore A23187 (specific for divalent cations and especially for Ca^{2+}) bypasses these intracellular steps by transporting extracellular calcium into the platelets or translocating intracellular calcium. When mammalian platelets are aggregated by adding ionophore, the same pattern of lithium effects is produced as in aggregating platelets with ADP (Table III).

TABLE III

Aggregation of mammalian platelets induced by ionophore A23187 after incubation with 10 mM LiCl for 90 minutes.

Species	Aggregation after Li incubation	µg ionophore/ ml PRP
MAN	200 units	2 µg/ml
MONKEY	96 units	3 µg/ml
COW	104 units	4 µg/ml
CAT	157 units	4 µg/ml
DOG	84 units	3 µg/ml
SHEEP	56 units	4 µg/ml
RABBIT	79 units	1.5 µg/ml
PIG	51 units	5 µg/ml

Control aggregation of PRP incubated with 10 mM NaCl was taken as 100 units of aggregation for each species.

DISCUSSION

Platelets display distinct species dependent properties, not only in quantitative but also in qualitative aspects (18). It therefore is doubtful whether conclusions about drug mechanisms obtained from animal platelets can be applied to human platelets.

This point of view is supported by our study, since we showed that the effects of lithium on the aggregation behaviour of mammalian platelets differ markedly (Fig. 1).

The observation that addition of lithium as well as of sodium can induce an instantaneous impairment of platelet aggregation (Fig. 2) pointsto an extracellular effect. This extracellular inhibiting effect slowly disappears upon prolonged incubation. As shown in Fig.2 in platelets from man and cat the long-term effect of lithium is a stimulation of aggregation. In bovine

and in monkey platelets lithium has no effect and in sheep, dog, rabbit and pig platelets it causes decrease of the aggregation.
The effects of lithium which only occur after incubation must be the result of intracellular events.

The long-term aggregation inhibition of rabbit platelets by lithium is counteracted by the addition of calcium (Table I). The same was observed by Anderson and Foulks (1), who supposed that lithium acts as a competitive inhibitor of calcium. This hypothesis, however, is not supported by results on human platelets, where calcium enhances the aggregation of lithium stimulated platelets (Table I).
Moreover, it is shown (Fig. 4) that the effect of lithium does not correlate with the calcium and/or magnesium content of platelets from different mammals. The large interspecies differences in intraplatelet calcium- and magnesiumcontents which we found are in agreement with published data (8,11,17,19,20). Although the total intraplatelet calcium concentration is not influenced by lithium, an interference with the process of the liberation of calcium into the cytoplasm, the area where calcium plays its role, is still possible.
With the present available techniques it is not possible to measure intracellular shifts of calcium by lithium. For this reason an indirect approach for investigating an intracellular interaction of lithium and calcium was used.
The ionophore A23187 induces membrane leakage for divalent cations especially calcium (15). This results in a rapid rise of the cytoplasmic calciumconcentration either by transport of extracellular calcium into the cell or by liberation of intracellularly stored calcium. This artificially induced rise in cytoplasmic calciumconcentration induces platelet aggregation. The effects of lithium on ionophore-induced aggregation are qualitatively the same as those on ADP-induced aggregation (Table III). This suggests that lithium does not interfere with the normal process of intracellular calcium liberation.

Also a displacement of calcium from storage sites during the incubation with lithium is not likely. Such a molecular displacement may be expected to act in the same way in platelets from all animals tested due to an increased cytoplasmic calciumconcentration. The divergency of the effects of lithium on aggregation of platelets of different species makes it unlikely that the action of lithium is restricted to a shift of calcium from intracellular stores.

Investigations from Frausto da Silva and Williams (5) suggest that lithium ions can interfere with calcium- or magnesium-dependent enzyme systems. The most probable site of action of lithium in platelets might be an inhibition of adenylate cyclase as shown for the stimulated enzyme (14,21). Another site of action may be an interference with prostaglandin synthesis e.g. with phospholipase A_2 which is calcium dependent (16). The effect of lithium on both enzyme systems deserves further study.

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CHAPTER 6

LITHIUM INHIBITS ADENYLATE CYCLASE OF HUMAN PLATELETS

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ABSTRACT

The ADP-induced aggregation of human platelets is markedly increased after preincubation with lithiumchloride, whereas lithium has the opposite effect on rabbit platelets. Since this phenomenon might be related to cAMP metabolism, the influence of lithium on total cAMP content and adenylate cyclase activity was investigated.
Lithium does not significantly change the total cAMP content of human platelets neither during incubation nor during ADP-induced aggregation.
Basal adenylate cyclase activity, however, is slightly inhibited by lithium in human platelets. The inhibition of adenylate cyclase induced by ADP appears to be enhanced, the stimulation of adenylate cyclase induced by prostacyclin appears to be counteracted by lithium.
In rabbit platelets the prostacyclin stimulated adenylate cyclase activity is not affected by lithium.
These data suggest that a correlation exists between the influence of lithium on the aggregation of human and rabbit platelets and the sensitivity of their adenylate cyclases for this ion.

INTRODUCTION

Platelet aggregation induced by adenosine diphosphate (ADP) is markedly changed after preincubation of platelets with millimolar concentrations of lithium ions. The aggregation magnitude is increased in human platelets and decreased in rabbit platelets (1-3).

The mechanisms of these effects of lithium are not clear. Although a slight increase of the release reaction occurs, we could show that this phenomenon cannot explain the increase of aggregation in human platelets (2).

Anderson and Foulks (4) suggested a competition between lithium and calcium as an explanation for the aggregation inhibition observed in rabbit platelets.

We showed that this hypothesis does not hold since the effects of lithium on the aggregation of various mammalian platelets are independent from their different calcium contents and since calcium has the same effect on the aggregation of lithium inhibited and lithium stimulated platelets (3).

It has been shown that lithium inhibits adenylate cyclase in different cell types, particularly after stimulation of the enzyme (5). In human platelets the basal enzyme activity is reported to be unaffected by lithium, whereas stimulated adenylate cyclase appeared to be inhibited (6,7). A relation between platelet function and the effect of lithium on adenylate cyclase activity is not yet demonstrated.

In general, stimulation of adenylate cyclase and/or inhibition of phosphodiesterase are accompanied by impaired platelet function (8,9). Aggregating agents, e.g. ADP, have no effect on platelet phosphodiesterase (10), whereas the reported effects on adenylate cyclase are contradictory (10-17).

Since the total cAMP content of platelets is measured by subtracting the amount in platelet-poor-plasma from that found in platelet-rich-plasma, the reliability of this method is limited. Moreover, with this method no discrimination is made between metabolic and storage pool cAMP. In order to study the effect of lithium on cAMP synthesis, the conversion of labeled ATP into cAMP by adenylate cyclase was determined with and without

prostacyclin stimulation (18) of the enzyme.

MATERIAL AND METHODS

Preparation of platelet-rich-plasma

Human PRP was prepared as described previously (19) by centrifugation (20 min at 250 G) of citrated blood (1/10 volume of 3.8 % (w/v) trisodium-citrate) on a Ficoll-Isopaque cushion (specific density 1.070 g/ml).
In cases where the cAMP content had to be determined, PRP was obtained by differential centrifugation (10 min, 250 g), since Ficoll appeared to interfere with the cAMP binding protein.
Rabbit PRP was prepared by differential centrifugation in all cases, since the withdrawal of erythrocytes from PRP by the Ficoll-Isopaque method appeared to be insufficient.
Platelet-poor-plasma (PPP) was prepared by centrifugation of citrated blood at 4000 G for 10 min.

Determination of the cyclic AMP content of platelets

Cyclic AMP was measured with a protein binding method (20) (Boehringer kit). Samples of 1 ml PRP were incubated for 90 min with 10 mM NaCl or LiCl and then either deproteinized immediately or after 1 min of aggregation with 3 μM ADP. Deproteinization was achieved by boiling for 3 min after addition of 0.5 ml sodium acetate buffer (0.1 M, pH 4.0) including 6 mM EDTA. After centrifugation the cAMP content in the supernatant was determined and expressed in pmol/ml PRP. In parallel experiments the cAMP content of PPP samples was determined. The difference between PRP and PPP reflects the cAMP content of the platelets. In control experiments no effect of LiCl or NaCl on the protein binding method was observed up to 10 mM endconcentration.

Determination of ^{14}C-adenine uptake by platelets

PRP samples of 1.5 ml were incubated at 37°C for 90 min with respectively 10 mM NaCl or LiCl after addition of 0.15 μCi (U-^{14}C)-adenine (spec. activity 286 mCi/mmol; Amersham). As indicated under Results, 2 mM papaverine,an inhibitor of phosphodiesterase activity,was added in some experiments 5 min

before terminating the incubation. From each sample 0.5 ml was centrifugated (1 min at maximum speed in an Eppendorf table centrifuge) and the radioactivity was counted in the supernatant. From these data ^{14}C-adenine uptake was calculated.

Conversion of ^{14}C-adenine into ^{14}C-ATP

To the remaining PRP samples 0.2 ml 3N $HClO_4$ containing 2 mM unlabeled ATP was added. The tubes were left on ice for 15 min and were centrifugated at 4000 G for 10 min. From each sample 10 µl of the supernatant was spotted on Ecteola-cellulose thin layer plates (Marchery Nagel, Polygram Cel 300 Ecteola) and developed with 0.1 N HCl. The ATP spot was scraped off and the radioactivity determined. From these data and the adenine-uptake the percentage conversion of adenine into ATP was calculated.

Determination of adenylate cyclase activity of platelets

The adenylate cyclase activity was determined as described by Haslam (16).1.25 ml samples of PRP were incubated at 37°C for 90-120 min with 10 mM NaCl or LiCl and with 0.5 µCi (U-^{14}C)-adenine (spec. activity 286 mCi/mmol, Amersham). The (U-^{14}C)-adenine was purified before use by chromatography as recommended (16). 2 mM papaverine and/or aggregating agents were added as described under Results. From each PRP sample 0.25 ml was taken to determine the uptake of ^{14}C-adenine in the way as described before.

Cyclic AMP was isolated from the remaining 1 ml of PRP essentially as described by Haslam and Taylor (21). The samples were deproteinized by adding 0.2 ml of icecold 3 N $HClO_4$, including 10^4 dpm ^3H-cAMP to determine the recovery and 2 mM cold cAMP. After centrifugation the supernatants were purified on columns of 1 ml Dowex AG50-WX8 (H^+form, Serva) by elution with H_2O. The 3rd to 7th ml were collected, lyophilized and the residues dissolved in 20 µl H_2O. These samples were subjected to two-dimensional thin layer chromatography (22) on cellulose sheets (Machery Nagel, MN 300), in the first direction with butan-1-ol-acetone-acetic acid-14.8M NH_3-water(90:30:20:1:60 by volume) and in the second with isobutyric acid-1M NH_3-0.1M EDTA (125:75:2 by volume). The cAMP spots were cut out, extrac-

ted with H_2O and the radioactivity counted in a LKB 81000 Liquid Scintillation Counter, equipped for $^{14}C/^{3}H$ double labeling.

Determination of the effect of prostacyclin (PGI_2) on cAMP content of platelets

The effect of PGI_2 on the cAMP content of platelets was determined according to Best et al. (22). 2 ml of buffer (15 mM Tris-HCl pH 7.4, 120 mM NaCl, 4 mM KCl, 1.6 mM $MgSO_4$, 2 mM NaH_2PO_4, 10 mM glucose and 0.2 % BSA) and papaverine (2 mM endconcentration) were added to 4.5 ml of PRP which was previously incubated for 90 min at 37°C with 10 mM NaCl or LiCl. The reaction mixture also included 40 μM arachidonic acid and fresh rabbit aorta rings (30 mg/ml) prepared according to Moncada et al. (18). Samples of 0.5 ml were taken at appropriate time intervals, added to 0.25 ml NaAC-buffer (0.1 M, pH 4,0 including 6 mM EDTA) and boiled for 3 min. After cooling on ice and centrifugation the cAMP content of the supernatant was determined.

Measurement of the effect of prostacyclin on aggregation

The effect of prostacyclin was determined according to Bunting et al. (24). Rabbit aorta rings in different concentrations were incubated for indicated periods at 37°C in 1.5 ml 50 mM Tris-HCl (pH 7.5) containing 60 μM arachidonic acid. Of this incubation mixture 20 μl samples were added to 0.6 ml of PRP and incubated for another 3 min at 37°C before inducing aggregation. Aggregation was induced with 2 μM ADP in human PRP and 0.7 μM ADP in rabbit PRP and performed in duplicate.
The aggregation inhibition percentages were calculated from the decrease in maximal aggregation in comparison with controls incubated with 20 μl Tris-HCl.

RESULTS

The effect of lithium on total cAMP content

The effect of lithium on total cAMP content of human platelets was investigated before and during aggregation. The difference

TABLE I

Lack of effect of lithium on cyclic AMP content of human platelets

		no addition	10 mM NaCl	10 mM LiCl
Exp. 1	PPP	19.7 ± 1.4	19.6 ± 1.5	18.0 ± 1.1
	PRP	27.5 ± 4.0	25.1 ± 2.9	24.7 ± 0.4
	PRP + ADP	28.0 ± 2.4	26.0 ± 4.0	26.5 ± 1.9
Exp. 2	PPP	29.9 ± 3.0	29.7 ± 5.4	29.1 ± 1.3
	PRP	37.3 ± 5.1	34.6 ± 1.8	36.6 ± 2.9
	PRP + ADP	36.2 ± 4.1	34.0 ± 4.8	34.0 ± 4.2
Exp. 3	PPP	20.0 ± 1.4	20.4 ± 1.6	19.5 ± 1.7
	PRP	24.9 ± 1.6	24.7 ± 3.2	26.2 ± 2.0
	PRP + ADP	24.9 ± 2.0	25.6 ± 1.2	26.1 ± 3.3
Exp. 4	PPP	N.D.	9.4 ± 0.5	10.0 ± 0.9
	PRP		15.7 ± 1.0	15.7 ± 0.1
	PRP + ADP		15.1 ± 2.2	15.1 ± 0.8

1 ml Samples of PPP and PRP were incubated at 37°C for 90 min with 10 mM NaCl, 10 mM LiCl or without addition. Part of the samples were aggregated with ADP (2-5 µM) for 1 min.
All samples were deproteinised and assayed for cAMP as described in Methods. Each figure is the mean ± S.D. from at least two samples, each assayed in duplicate for cAMP (in pmol/ml PRP or PPP).

between the cAMP content of PRP and that of PPP reflects the cAMP content of the platelets. As is shown in Table I the cAMP content of PPP amounted to approximately 70 % of the total cAMP in PRP. No changes were observed in the cAMP content of platelets incubated with lithium compared to the sodium incubated samples. Also aggregation by ADP does not induce detectable changes in cAMP content.

The effect of lithium on adenylate cyclase activity

The method we used for measuring the adenylate cyclase activity is an indirect two-step reaction: 1) labeling of ATP from ^{14}C-adenine and 2) conversion of the labeled ATP into ^{14}C-cAMP. A prerequisite for this method is that the specific radioactivity of platelet ATP is not affected by lithium. Therefore we first studied the conversion of labeled adenine into ATP in controls and in samples with lithium chloride added. The total uptake of ^{14}C-adenine by sodium and lithium incubated samples was equal (approx. 80 %). Platelet samples incubated with sodium and lithium convert the adenine into ATP equally (Table II). Also papaverine, a phosphodiesterase inhibitor used in the determination of adenylate cyclase activity, had no effect on the conversion of labeled adenine into ATP. Since lithium has also no effect on the total ATP content of platelets (25), differences in ^{14}C-cAMP, if detected, cannot be explained by differences in labeled precursor ATP.

The basal conversion percentage of ATP into cAMP in different PRP samples usually amounted from 0.03 to 0.05 %. In normal platelets lithium nor ADP induced a change in the amount of ^{14}C-cAMP present after incubation with ^{14}C-adenine.

In the presence of a phosphodiesterase inhibitor the amount of ^{14}C-cAMP rises linearly for at least 1 minute and therefore is a real parameter for the basal adenylate cyclase activity(16). As is shown in Table III the basal adenylate cyclase activity is inhibited for 3-17 % by lithium compared with the sodium incubated controls. The inhibition of adenylate cyclase during ADP induced aggregation of lithium incubated samples is even more pronounced. The lithium incubated samples showed 11 to 65 % inhibition, the control samples 4-47 % inhibition of adenylate cyclase.

Effect of prostacyclin on total cAMP content and aggregation of human platelets

The inhibitory action of lithium on human platelet adenylate cyclase is much more obvious when the enzyme is stimulated

TABLE II

Conversion of ^{14}C-Adenine into ^{14}C-ATP by human platelets

10 mM NaCl	: 78.7 ± 7.3
10 mM NaCl + 2 mM papaverine	: 77.5 ± 6.7
10 mM LiCl	: 79.7 ± 5.7
10 mM LiCl + 2 mM papaverine	: 80.1 ± 3.6

Platelets were incubated with 0.15 µCi ^{14}C-adenine/ml PRP.
Uptake and conversion into ^{14}C-ATP were measured as described under Methods.
Conversion is expressed as percentage of the adenine taken up (mean ± S.D.: n = 4).

TABLE III

Effect of lithium on adenylate cyclase activity of human platelets.

	Percentage inhibition		
	10 mM Li	10 mM Na + ADP	10 mM Li + ADP
Exp. 1:	11 %	35 %	41 %
Exp. 2:	3 %	5 %	11 %
Exp. 3:	10 %	4 %	20 %
Exp. 4:	17 %	47.5 %	65 %
Exp. 5:	6 %	12 %	18 %

PRP samples were incubated with 10 mM NaCl or LiCl for 90-120 minutes at 37°C. ^{14}C-Adenine was included too in this incubation. Adenylate cyclase activity was determined as described under Methods as the difference in conversion of ^{14}C-Adenine into ^{14}C-cAMP in PRP samples, before and after incubation with 2 mM papaverine for 1 min.
In some samples ADP was also added for this period. The percent inhibition of adenylate cyclase was calculated from the adenylate cyclase activity in samples incubated with 10 mM NaCl as control (0 % inhibition).

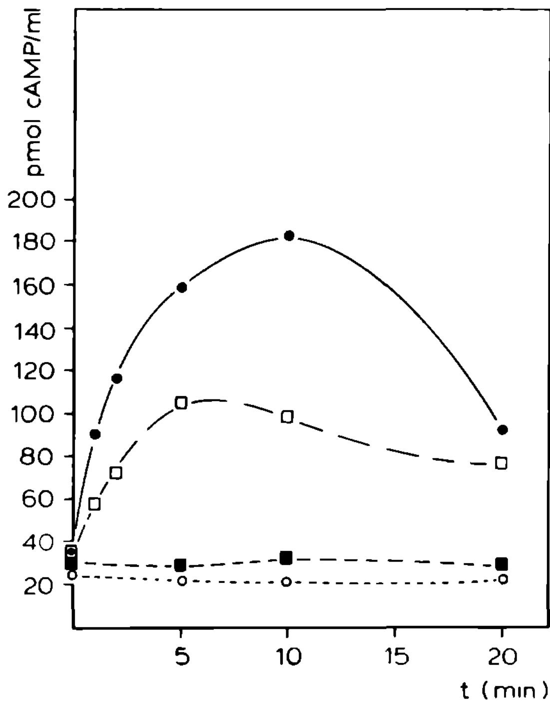

FIGURE 1

Effect of prostacyclin on cyclic AMP content of human platelets.

PRP was incubated with 10 mM NaCl or LiCl for 90 min. Increase of cAMP content by prostacyclin production in situ was measured as described under Methods and plotted against the time of incubation with aorta rings.

■ : PRP - aorta

○ : PPP + aorta

● : PPP + 10 mM NaCl + aorta

□ : PRP + 10 mM LiCl + aorta

by prostacyclin. As is demonstrated in Fig. 1 the rise in cAMP content in lithium incubated samples is slower and the maximal cAMP content much lower than in controls. Control experiments with PPP and PRP without prostacyclin show that the cAMP content remains fairly constant during the incubation time when respectively no platelets, or no prostacyclin is present. The stimulation of platelet adenylate cyclase by prostacyclin causes an inhibition of the ADP induced aggregation in human platelets (Table IV).

TABLE IV

Inhibition by prostacyclin of ADP-induced aggregation of human platelets

		Inhibition of aggregation (perc.) Incubation times of aorta rings		
		3 min	6 min	9 min
10 mg aorta	Na	20 %	35 %	N.D.
	Li	10 %	-20 %*	N.D.
20 mg aorta	Na	80 %	75 %	65 %
	Li	30 %	10 %	0 %
50 mg aorta	Na	95 %	95 %	93 %
	Li	60 %	60 %	58 %

PRP samples of 0.6 ml were preincubated for 90 min with 10 mM NaCl or LiCl at 37°C.
Rabbit aorta rings were incubated at 37°C for indicated periods in 1.5 ml 50 mM Tris-HCL pH 7.4. 20 µl of this incubation mixture was added to the PRP and left at 37°C for 3 min before inducing aggregation with ADP (2 µM). The decrease in O.D. max. in comparison with controls (Na - and Li- incubated samples without prostacyclin) was taken as percentage inhibition.
*Stimulation of aggregation.

The extent of inhibition correlates with the amount of aorta used and with the incubation time in the buffer.
In human platelets the inhibition of aggregation is antagonized by preincubation with lithium (Table IV). The amount of inhi-

bition (20 mg aorta/incubation mixture; 3 min of incubation) ranged in 4 experiments from 25 to 35 % in lithium incubated samples and in controls from 70 to 80 %.

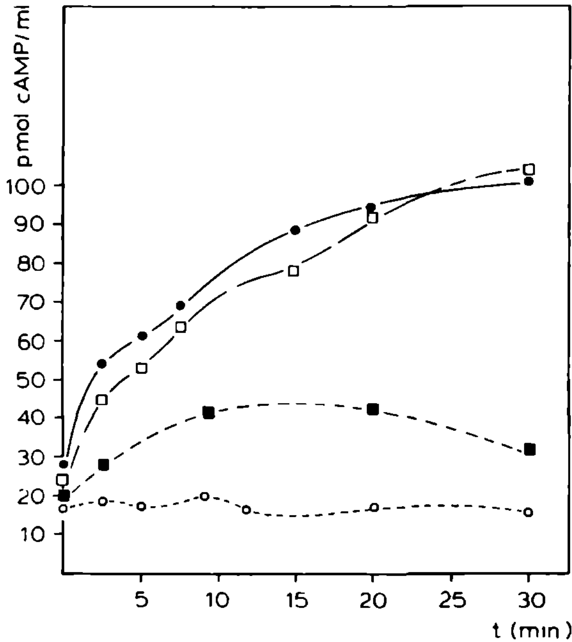

FIGURE 2

Effect of prostacyclin on cyclic AMP content of rabbit platelets
For explanation see legend to Figure 1.

Effect of prostacyclin on total cAMP content and aggregation of rabbit platelets

Because lithium does not stimulate but inhibits the aggregation of rabbit platelets the effect of lithium on cAMP metabolism of rabbit platelets was also determined. Since basal adenylate cyclase activity is very low, the effect of lithium on adenylate cyclase activity could only be measured after stimulation with prostacyclin. As shown in Fig. 2 the influence of lithium

on the stimulated adenylate cyclase activity in rabbit platelets is completely different compared to that on human platelets. Lithium and sodium incubated samples show an almost equal increase in cAMP content, indicating that lithium in this concentration does not affect the stimulated adenylate cyclase of rabbit platelets. The stimulation of platelet adenylate cyclase by prostacyclin causes an inhibition of rabbit platelet aggregation (Table V). However, in contrast to human platelets, lithium does not antagonize the aggregation inhibition (Table V). The amount of inhibition in rabbit platelets ranges from 33 to 50 % for both lithium incubated samples and controls in 4 experiments (20 mg aorta/incubation mixture, 3 min of incubation).

TABLE V

Inhibition by prostacyclin of ADP-induced aggregation of rabbit platelets

		Incubation times of aorta rings	
		1 min	3 min
10 mg aorta	Na	17 %	38 %
	Li	15 %	33 %
20 mg aorta	Na	26 %	41 %
	Li	29 %	36 %
50 mg aorta	Na	62 %	50 %
	Li	52 %	47 %

Procedure was the same as described in Table IV except that aggregation was induced with 0.7 μM ADP.

DISCUSSION

Cyclic AMP is probably stored in the platelet in various compartments of which only a small part plays a regulatory role (26). It is therefore not surprising that lithium does not change significantly the total cAMP content of human platelets (Table I). The observation that ADP-induced aggregation did not decrease the cAMP content of platelets (Table I) is in

contrast with the results of Salzman (13-15), but is in agreement with the data of other investigators (10-12, 16).
Basal adenylate cyclase activity in human platelets was determined from the conversion of (U-^{14}C)-adenine via ^{14}C-ATP into ^{14}C-cAMP, as well in the absence as in the presence of papaverine, an inhibitor of phosphodiesterase. The very low basal conversion of ATP into cAMP in the absence of papaverine (0,03-0,05 %) and the lack of effect of ADP on this basal conversion, are in agreement with the findings of Haslam (16). Preincubation with lithium does not affect this basal conversion of ATP into cAMP.
When platelet phosphodiesterase is inhibited by papaverine, lithium appears to inhibit slightly the accumulation of cAMP (Table III). During aggregation with ADP the inhibition of this basal adenylate cyclase activity amounts 4 to 47 %, which is in agreement with the observations reported by Haslam (16).
In lithium incubated samples the inhibition of platelet adenylate cyclase during ADP-induced aggregation is more pronounced than in controls (11-65 %). These results are in agreement with the recently described inhibition of adenylate cyclase by ADP in a purified human platelet plasma membrane preparation (17).
In vivo platelet functioning is partly regulated by prostacyclin, the strongest and most physiological aggregation inhibitor (10,27). Prostacyclin acts through stimulation of adenylate cyclase, thus raising the intracellular cAMP content (23, 28, 29). In Fig. 1 is shown that the stimulation of adenylate cyclase by prostacyclin in human platelets is antagonized by lithium. The results shown in Table IV demonstrate that the inhibition of ADP-induced aggregation of human platelets by prostacyclin is counteracted by lithium.An inhibition of stimulated adenylate cyclase by lithium has also been reported by other investigators (6,7). Their failure to detect an inhibition of the basal enzyme activity by lithium is probably due to the different methods used.
Lithium also interferes with adenylate cyclase activities in other types of cells (5), probably by replacing magnesium in the enzyme-magnesium complex, a common theory for the action

of lithium (30). However, this phenomenon might be species specific as was observed for other enzymes (31).
We reported previously that lithium does not stimulate but inhibits the ADP-induced aggregation of rabbit platelets (3). From Fig. 2 it can be concluded that the stimulation of adenylate cyclase by prostacyclin in rabbit platelets is not inhibited by lithium. In Table V it is shown that the inhibition of ADP-induced aggregation of rabbit platelets by prostacyclin is hardly affected by lithium.
In view of these results it seems likely that the enhancement of human platelet aggregation by lithium is achieved by inhibition of adenylate cyclase activity. Other adenylate cyclase inhibitors show a more inconsistent effect on platelet aggregation (32,33).

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CHAPTER 7

LITHIUM STIMULATES THROMBOXANE B_2 FORMATION IN HUMAN PLATELETS

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ABSTRACT

The aggregation of human platelets is enhanced after preincubation with lithium salts. Previously it was shown that lithium inhibits adenylate cyclase activity in human platelets.
Enhancement of aggregation and inhibition of adenylate cyclase by lithium was not observed in rabbit platelets.
In this paper it is shown that in human platelets lithium enhances considerably the synthesis of thromboxane B_2, whereas the synthesis of PGE_2 and $PGF_{2\alpha}$ was enhanced to a lesser extend. In rabbit platelets lithium had no effect on prostaglandin synthesis. It is concluded that lithium specifically stimulates the synthesis of thromboxanes in human platelets, probably due to the previously reported inhibition of adenylate cyclase activity, resulting in an increased aggregability.

INTRODUCTION

Unstable metabolites of arachidonic acid, prostaglandin endoperoxides (1,2) and thromboxanes (3), play a regulatory role in platelet function. The cyclic AMP system is also involved in platelet function,in as far raising the cAMP content of platelets results in a decrease of aggregation (4,5),a lowering of the availability of arachidonic acid to prostaglandin synthesis (6-8) and possibly in an inhibition of the cyclooxygenase system (9,10).

We observed that preincubation with lithium causes a stimulation of the aggregation of human platelets, together with an inhibition of the platelet adenylate cyclase activity (11). This inhibition of adenylate cyclase and stimulation of aggregation was not observed in rabbit platelets (11).

In order to establish the role of lithium in the relationship between platelet aggregation, adenylate cyclase activity and prostaglandin synthesis, the effects of lithium on prostaglandins PGE_2 and $PGF_{2\alpha}$ and thromboxane B_2 (TxB_2) formation were studied in as well human as rabbit platelets.

MATERIAL AND METHODS

Preparation of Platelet-Rich Plasma (PRP)

Human PRP was prepared by centrifugating citrated blood (1 part of 3,8 % (w/v) trisodiumcitrate on 9 parts blood) on a Ficoll-Isopaque cushion for 20 min at 250 G (12). Rabbit PRP was prepared by differential centrifugation (10 min, 300 G) of citrated blood (13).

PRP was preincubated for 90-120 min at 37°C with 10 mM LiCl or 10 mM NaCl (as control).

Aggregation

Aggregation in PRP was measured photometrically (14) with modifications as described (13). Aggregation was induced with arachidonic acid (Sigma), diluted to appropriate concentrations in

absolute ethanol under nitrogen just before use. In the experiments on TxB_2 generation, aggregation was also induced with ADP and collagen, both in Veronal buffer (0.15 M, pH 7.2).

Preparation of samples for prostaglandin synthesis studies

In PRP samples incubated with Na^+ or Li^+, the prostaglandin concentrations were determined either before or after 3 min of aggregation, induced by different agents. The plasma samples were prepared according to Thomas et al. (15,16). In short, 1 ml PRP samples were acidified to pH 3.5 and small amounts (10^3 DPM) of $^3H-PGF_{2\alpha}$ were added for recovery determinations. The samples were extracted twice with 5 ml of ethyl acetate and the combined organic layers were dried and the residues taken up in 1 ml of water. These solutions were applied to Sephadex G-25 columns (Pharmacia) and eluted with distilled water. The low molecular mass effluents containing the prostaglandins, were collected, dried and dissolved in assay buffer (50 mM Tris -HCl, pH 7.4, containing 150 mM NaCl and 0.1 % (w/v) gelatin). Control experiments with labeled $PGF_{2\alpha}$, PGE_2 and TxB_2 revealed that the procedural losses observed were equal and always less than 40 %.

Preparation of ^3H-Thromboxane B_2

$^3H-TxB_2$ was prepared by a combined incubation of seminal vesicle microsomes and platelet microsomes with ^3H-arachidonic acid (Amersham, 135 Ci/mmol). Seminal vesicle microsomes were prepared according to Flower et al. (17) and platelet microsomes according to Ho et al. (18). Seminal vesicle microsomes, suspended in 50 mM Tris-HCl (pH 8.0) including 2 µM hemoglobin and 5 mM L-tryptophan, were incubated at 37°C with ^3H-arachidonic acid in a final concentration of 10 µCi/ml.
After 2 min platelet microsomes, suspended in 50 mM Tris-HCl (pH 6.8) and including 2 mM reduced glutathion, were added and the incubation continued for 20 min. The reaction was stopped by acidifying to pH 3 with HCl and the mixture was extracted three times with 5 volumes of ethyl acetate. After drying on $MgSO_4$ and evaporating the solvent the residue was taken up

in ethyl acetate and subjected to chromatography on silicagel plates, which were developed with chloroform-methanol-acetic acid (90:5:5 v/v/v) according to Ho et al. (18). The $^3H-TxB_2$ fraction was cut off, eluted and dissolved in assay buffer. Unlabeled TxB_2 was prepared in a similar way from unlabeled arachidonic acid (Sigma) with negligible amounts of ^3H-arachidonic acid added for estimating the conversion percentage.

RIA procedure

The procedures of the radioimmunoassays (RIAs) for $PGF_{2\alpha}$ and PGE_2 were described by Thomas et al. (15,16). The RIA for TxB_2 was developed according to the same technique. The antiserum against TxB_2 was kindly provided by dr F.A.Fitzpatrick (The Upjohn Company, Kalamazoo, Mich. USA) who described the specificity of this antiserum (20). His RIA procedure and the technique introduced here showed almost identical standard-dose response relationships for TxB_2; minimum detectable quantity amounted 0.2-0.4 ng TxB_2 standard per tube.

RESULTS

Arachidonic acid induced aggregation

Incubation with 10 mM LiCl for 90 min enhanced the arachidonic acid induced aggregation of human platelets (Table I). The minimum concentration of arachidonic acid required to induce aggregation, was lower in lithium incubated samples than in controls. The concentration at which maximal aggregation was obtained was also lower in lithium incubated samples.

Prostaglandin formation

Preliminary experiments revealed that in the RIA for thromboxane B_2 the procedure with Sephadex chromatography produced better parallelism than the dilution technique of Fitzpatrick et al. (19) although at higher sample quantities linearity was not maintained perfectly.

TABLE I

Effect of lithium on arachidonic acid induced aggregation of human platelets

Conc. of arachidonic acid	Units aggregation	
	10 mM NaCl	10 mM LiCl
0.10 mM	0	6
0.13 mM	5	86
0.16 mM	7	95
0.19 mM	12	98
0.21 mM	85	100*
0.22 mM	98	

* Results are from one typical experiment

Maximal aggregation defined as 100 units of aggregation

The effects of lithium preincubation on human platelet prostaglandin formation are shown in Table II. Without aggregation induction the concentrations of TxB_2, PGE_2 and $PGF_{2\alpha}$ in lithium incubated samples did not differ from those in NaCl incubated controls.

Aggregation with ADP did not induce substantial prostaglandin synthesis, neither in sodium- nor in lithium chloride incubated samples.

During collagen induced aggregation the platelet thromboxane B_2 synthesis was higher in lithium incubated than in sodium incubated PRP. A less pronounced effect was seen on the synthesis of the two prostaglandins PGE_2 and $PGF_{2\alpha}$.

During aggregation induced by arachidonic acid, the TxB_2 formation was also increased in lithium incubated samples compared to controls. The experiments on human platelets as shown in Table II were repeated three times and analysed by the paired T-test. It appeared that after aggregation with an arachidonic acid concentration of 1.2 mM, the contents of TxB_2 in nanograms/ml PRP after sodium incubation ranged from 450 to 750 and in lithium incubated samples > 1900 ($p < 0.01$).

TABLE II

Concentration of Thromboxane B_2, PGE_2 and $PGF_{2\alpha}$ in human PRP, incubated with 10 mM NaCl or 10 mM LiCl

	TxB_2		PGE_2		$PGF_{2\alpha}$	
	10 mM NaCl	10 mM LiCl	10 mM NaCl	10 mM LiCl	10 mM NaCl	10 mM LiCl
No aggregation	8	8	0.20	0.20	0.39	0.40
1 μM ADP	9	9	0.23	0.29	0.34	0.40
4 μM ADP	10	9	0.18	0.28	0.34	0.35
5 μg collagen/ml	20	29	0.73	0.91	0.46	0.51
10 μg collagen/ml	82	425	0.98	1.50	0.70	1.28
0.8 mM arachidonic acid	431	784	27	31	11.5	13.5
1.2 mM arachidonic acid	745	1930	45	45	24.3	24.5

Platelets were incubated for 90 min with 10 mM NaCl or 10 mM LiCl at 37°C, before inducing aggregation. PRP samples were processed as described in methods. Results are given from one typical experiment. All concentrations are in nanograms/ml PRP.

The concentration of the prostaglandins PGE_2 and $PGF_{2\alpha}$ was much higher now than during collagen induced aggregation, but almost no differences were observed between lithium incubated samples and controls.

In rabbit platelets no differences were observed in the TxB_2, PGE_2 or $PGF_{2\alpha}$ concentrations directly after incubation with either lithium or sodiumchloride or during aggregation (Table III). The measurements with arachidonic acid induced aggregation (0.8 mM) from which one example is shown in Table III were repeated four times. In sodium incubated rabbit platelet samples the thromboxane B_2 content ranged from 890 to 1200 and in lithium incubated samples from 900-1150 nanograms/ml PRP ($p > 0.3$). Lithium had no effect on arachidonic acid induced aggregation of rabbit platelets and only a slight inhibitory effect on ADP and collagen aggregation.

DISCUSSION

Human platelets exhibit an increased aggregation potency after incubation with lithiumchloride.This phenomenon was observed after aggregation induction with ADP, collagen, thrombin, adrenalin and ionophore A23187 (13,20,21). The influence of lithium on aggregation appeared to differ markedly for different mammalian species, e.g. lithium has no effect or even diminishes the aggregation potency of rabbit platelets (13). Till now no satisfying explanation for these effects of lithium has been given.

Human platelets in which the cAMP level is elevated show a diminished aggregation potency, whereas it is suggested that a lowering of the cAMP level may result in an increased aggregation potency. Since lithium lowers the cAMP content in certain tissues (e.g. brain and thyroid), Geerdink et al. (20) proposed that the effect of lithium on aggregation could be explained by an inhibition of adenylate cyclase activity in human platelets. A lowering of the cAMP level might also,due to activation of phospholipase A_2, increase the availability of arachi-

TABLE III

Concentration of Thromboxane B_2, PGE_2 and $PGF_{2\alpha}$ in rabbit PRP, incubated with 10 mM NaCl or 10 mM LiCl

	Thromboxane B_2		PGE_2		$PGF_{2\alpha}$	
	10 mM NaCl	10 mM LiCl	10 mM NaCl	10 mM LiCl	10 mM NaCl	10 mM LiCl
No aggregation	1.3	0.8	0.01	0.01	0.43	0.39
1 μM ADP	2.6	1.7	0.03	0.04	0.45	0.41
15 μg collagen/ml PRP	35	42	0.13	0.13	0.66	0.74
0.8 mM arachidonic acid	1000	910	12	14	30	30

Platelets were incubated for 90 min with 10 mM NaCl or LiCl at 37°C before inducing aggregation. PRP samples were processed as described in methods. Results are given from one typical experiment. All concentrations are in nanograms/ml PRP.

donic acid to the synthesis of prostaglandins (6-8).
We reported previously that lithium inhibits basal and stimulated adenylate cyclase activity in human platelets and has no effect on this enzyme system in rabbit platelets (11).
This paper describes the effect of lithium on the synthesis of PGE_2, $PGF_{2\alpha}$ and TxB_2 in human and rabbit platelets that show completely different effects of lithium on the aggregation pattern.
It appears that lithium incubation as such does not change the basal levels of PGE_2, $PGF_{2\alpha}$ and TxB_2 in human platelets. During aggregation, induced with collagen or arachidonic acid the synthesis of PGE_2 and $PGF_{2\alpha}$ is stimulated equally in human platelets incubated with lithium chloride or sodium chloride. The synthesis of TxB_2, however, is considerably stimulated by lithium when human platelet aggregation is induced by collagen or arachidonic acid (Table II).
In rabbit platelets, in contrast to human platelets, lithium has no effect on the synthesis of PGE_2, $PGF_{2\alpha}$ and TxB_2 during aggregation, induced by the various stimules (Table III).
The observed basal levels of TxB_2 in human platelets are in agreement with the data of Fitzpatrick et al. (19,22). The amounts of TxB_2 which were found during aggregation are much higher than reported by them (19,22). This discrepancy may be due to differences in the methods used.
From the foregoing data we conclude that lithium enhances aggregation by inhibition of adenylate cyclase resulting in an increased thromboxane formation. The lack of influence of lithium on adenylate cyclase activity and thromboxane synthesis in rabbit platelets, together with a lack of effect on aggregation, supports strongly the proposed mechanism of lithium action.

The fact that also ADP induced aggregation in human platelets is enhanced after lithium incubation, though thromboxane synthesis is not stimulated, shows that the influence on thromboxane synthesis is a secondary effect. The inhibition of adenylate cyclase plays apparently a key role in the effect of lithium on human platelet aggregation.

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SAMENVATTING

Dit proefschrift handelt over biochemisch onderzoek aan bloedplaatjes dat werd verricht naar aanleiding van de waarneming dat lithium de aggregatie van humane bloedplaatjes stimuleert. De effekten van lithium op bloedplaatjes werden nader gekarakteriseerd en de invloed van lithium op diverse biochemische regulatiemechanismen van m.n. humane bloedplaatjes werd bestudeerd.
In het eerste hoofdstuk wordt een overzicht gegeven van de huidige kennis betreffende de funktie en de biochemische processen van bloedplaatjes. Hierbij komen vooral ter sprake de verschillende processen die de bloedplaatjesfunktie besturen, te weten: intracellulaire verschuivingen van calcium, de spiegel van cyclische nucleotiden en de synthese van prostaglandine endoperoxiden/thromboxanes.
In het tweede hoofdstuk worden enige relevante chemische en biochemische eigenschappen van lithium beschreven. De overeenkomsten en verschillen tussen lithium en andere ionen, m.n. natrium/kalium en calcium/magnesium worden besproken. Tevens worden in dit hoofdstuk de effekten van lithium op verschillende regulatiemechanismen in cellen, het cyclisch AMP en het intracellulair calcium, behandeld. Bovendien worden de tot nu toe bekende effekten van lithium op bloedplaatjes, waaronder de stimulatie van de aggregatie beschreven.
In hoofdstuk 3 wordt een verbeterde methode om plaatjesrijk plasma te bereiden m.b.v. centrifugatie op een Ficoll-Isopaque kussen beschreven. Deze methode bleek uiterst nuttig te zijn bij het merendeel van de volgende experimenten.
Hoofdstuk 4 behandelt de experimenten waarmee de stimulatie van de aggregatie van humane plaatjes door lithium werd gekwantificeerd. De invloed van verschillende koncentraties lithium en incubatietijd op de ADP-geinduceerde aggregatie werd onderzocht. Ook de stimulatie van de serotonine-release werd gemeten. Het bleek dat de effekten van lithium op de plaatjesfunktie niet verklaard kunnen worden door een toegenomen release mechanisme alleen. Wel werd een stimulatie van de release

waargenomen.
In hoofdstuk 5 zijn de uiteenlopende effekten van lithium op de aggregatie van bloedplaatjes bij verschillende zoogdieren beschreven.
Deze effekten varieren van een stimulatie bij sommige diersoorten tot een remming bij andere, terwijl bij een paar diersoorten vrijwel geen effekt wordt gezien. Deze diversiteit van de waargenomen effekten wijst op de verschillende wijzen waarop lithium in verschillende dierplaatjes werkt. Een in de literatuur voorgesteld mechanisme voor de werking van lithium, een specifieke interaktie met extracellulair calcium, werd weerlegd. In zowel lithium gestimuleerde (humane)als lithium geremde (konijne) plaatjes blijkt calciumtoevoeging eenzelfde stimulatie van de aggregatie te veroorzaken.
Aangezien aggregatie induktie met calciumionofoor in humane en konijneplaatjes dezelfde species-specifieke effecten van lithium vertoont, is een interaktie van lithium met intracellulair calcium onwaarschijnlijk.
In hoofdstuk 6 worden de effekten van lithium op het cyclisch AMP regulatiestelsel van humane en konijne bloedplaatjes beschreven. Ofschoon lithium geen verlaging van het totaal cyclisch AMP gehalte van bloedplaatjes veroorzaakt, blijkt het in humane plaatjes wel het cyclisch AMP synthetiserend enzym, adenylaat cyclase, te remmen. Dit is vooral duidelijk merkbaar als dit enzym tevens gestimuleerd wordt met prostacycline, dat een sterk aggregatie remmende werking heeft. De remming van door prostacycline gestimuleerd adenylaat cyclase was in konijne plaatjes niet aantoonbaar. De effekten van lithium op de aggregatie van humane bloedplaatjes zijn in overeenstemming met het effekt op het adenylaat cyclase: de remming van adenylaat cyclase door lithium gaat gepaard met een stimulatie van de aggregatie en een tegengaan van het aggregatieremmende effekt van prostacycline. In konijneplaatjes past het ontbreken van het effekt van lithium op het adenylaat cyclase bij het gelijk blijven van het effekt van prostacycline; voor het licht aggregatieremmend effekt van lithium alleen moet een ander, tot nu toe onbekend, mechanisme verondersteld worden.

Aangezien beinvloeding van het cyclisch AMP systeem ook gevolgen heeft voor de synthese van prostaglandine endoperoxides en thromboxanen, worden de effekten van lithium op de aanmaak van deze sterk aggregatiebevorderende metabolieten in hoofdstuk 7 beschreven. Zoals verwacht blijkt lithium deze synthese in humane plaatjes te stimuleren, mits natuurlijk de aggregatie geinduceerd wordt met stoffen die via het prostaglandine systeem werken, zoals arachidonzuur. In konijneplaatjes wordt dit effekt niet waargenomen.

Aangezien lithium ook de aggregatie stimuleert die geinduceerd wordt door stoffen die niet via het prostaglandine systeem werken, zoals bijvoorbeeld ADP, is remming van de adenylaat cyclase aktiviteit toch het meest waarschijnlijke mechanisme waarlangs lithium de aggregatie van humane bloedplaatjes bevordert.

CURRICULUM VITAE

Leon Imandt werd geboren op 31 januari 1951 te Bergen op Zoom. Hij bezocht het Mollerlyceum te Bergen op Zoom en behaalde daar het diploma Gymnasium β in 1969. In hetzelfde jaar werd begonnen met de studie Scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen S_2 (hoofdvak scheikunde met bijvakken wiskunde, natuurkunde en biologie) werd afgelegd in september 1972. In mei 1975 werd het doctoraalexamen afgelegd met als hoofdrichting biochemie (Prof.Dr.H.Bloemendal), als bijvakken Organische Chemie (Prof.Dr.R.Nivard) en Waterzuivering (prof.Dr.P.Fohr, Landbouwhogeschool te Wageningen).
Van februari 1975 tot september 1978 was hij werkzaam als wetenschappelijk medewerker in FUNGO/ZWO dienst op het hematologisch laboratorium van de afdeling Inwendige Geneeskunde, St.Radboud Ziekenhuis te Nijmegen, waar dit proefschrift bewerkt werd. Sinds 1 september 1978 is hij werkzaam op het Hematologisch Laboratorium van het Akademisch Ziekenhuis van de Vrije Universiteit te Amsterdam.

STELLINGEN

1

Konklusies over het werkingsmechanisme van pharmaca in menselijke bloedplaatjes kunnen niet getrokken worden uit experimenten met bloedplaatjes van andere zoogdieren.

Dit proefschrift.

2

In de door Anderson en Foulks voorgestelde hypothese aangaande een interaktie van lithium en calcium,als verklaring voor het aggregatie-remmend effekt van lithium op konijne bloedplaatjes, wordt geen rekening gehouden met het stimulerende effekt van calcium op de aggregatie.

Anderson & Foulks:Thromb.Haemostas.36,343,1976.
Dit proefschrift.

3

In de effekten van lithium op menselijke bloedplaatjes neemt de remming van adenylaat cyclase een sleutelpositie in.

Dit proefschrift.

4

Een dosering van 2 maal daags 80 mg. aspirine,zo nodig na een eerste oplaad-dosering van 500 mg.,is de meest optimale voor het remmen van de bloedplaatjesfunktie bij de mens.

P.C.Huygens et al.:Scand.J.of Haemat.,in press.

5

Het bepalen van glucocorticoid receptors in leukemische cellen kan ook nuttig zijn voor de keuze van therapie bij patiënten met akute myeloide leukemie.

E.B.Thompson:Canc.Treatment Rep.63,104,1979.

6

Voor de klassifikatie van akute leukemiën is de kleuring op niet-specifieke esterasen onontbeerlijk.

7

De indeling van akute myeloide leukemiën volgens de FAB-klassifikatie,m.n. het onderscheid tussen M1 en M2 en tussen M5A en M5B,kan verbeterd worden door gebruik te maken van de "rijpingsindex".

D.J. van Rhenen et al.:Brit.J.of Haemat.,in press.

8

De inspanningen en risiko's van pogingen om kontinue normoglycemie te bereiken bij patiënten met diabetes mellitus,teneinde de progressie van vaatafwijkingen te vertragen,zijn slechts gerechtvaardigd indien roken en orale contraceptiva worden gestaakt,gelet op de effekten hiervan op de vetstofwisseling.

G.D.Calvert et al.:Lancet,ii,66,1978.
A.C.Artzenius et al.:Lancet,i,1221,1978.

9

De stelling dat deeltijdarbeid in leidinggevende funkties niet mogelijk is,is onbeproefd en dus onbewezen.

10

De verruwing van het Nederlandse voetbal kan het best bij de wortel worden aangepakt,d.w.z. bij de begeleiders van pupillenvoetbal.

11

Those Bloody Platelets !

Nijmegen, 9 oktober 1980 Leon Imandt