New perspectives in blood platelet aggregation

M. Jamaluddin
Thrombosis Research Unit, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram 695 012, India

Aggregation of blood platelets is indispensable for haemostasis but fraught, in some circumstances, with pathological consequences whose mechanism-based prevention and treatment are a long-sought-after goal that remains elusive partly because of a lack of understanding of mechanisms. A glimmer of new insight is emerging. Aggregation appears to involve stimulus-induced, energy-dependent creation of heterogeneous platelet species which sort out partners by long-range, possibly hydrophobic, attractive forces and cell-adhesion receptor counter-receptor discriminating reactions. Dynamic extrusion of pseudopods with reactive cell-adhesion receptors on them appears to be essential. Aggregation is first order and occurs without significant compression or interdigitation of glycolalyses and cooperativity forms an important aspect of its modulation. Platelets could form a convenient model system to study intercellular interactions.

Platelets are small anucleate excitable cells derived from megakaryocytes of bone marrow. In circulation, hydrodynamic properties of the larger and more abundant flowing red cells keep platelets in close contact with the vessel wall where they have many functions.

Their most important function by far is in haemostasis, that is, arrest of bleeding from a vascular wound. It is achieved by their adhering to the wound and precipitating the formation and consolidation of a haemostatic plug that seals the wound. The smooth and safe conduct of this process requires normal functioning of not only platelets but also of plasma factors, the vessel and other blood cells. Platelet functioning in other circumstances is fraught with pathological consequences requiring prevention and treatment.

The essence of platelet functioning is their excitability, that is, their ability to recognize, respond to and be activated, in specific ways, by special, external, physiological stimuli (agonists) which act on their respective cell surface receptors. But paradoxically, platelets may also be activated in vitro by a growing number of certain types of chemical and physical entities (Table 1) which act receptor-independently, a phenomenon not without its practical applications as, for example, in investigating the biochemical mechanisms of platelet activation or in assessing blood compatibility of biomaterials.

Activation triggers a few interconnected and interacting biochemical pathways that provide mechanisms for fast morphological changes of platelets (shape-change), their sticking and spreading on surfaces (adhesion), sticking to one another (aggregation) as well as their release of biologically active and functionally important molecules stored in special granules (secretion). None of these reactions is a simple one-step process but all of them have physiological relevance, for haemostasis, inflammatory reactions, fibrinolysis and promotion of wound healing although their temporal sequence or cause-effect relationships are not clearly delineated.

Physiologically aggregation and secretion follow shape-change reactions. But in vitro, and probably in vivo also, platelets can be activated to varying degrees and stages depending on the nature, dose and duration of action of the stimuli, on the one hand, and the number,
Table 1. Platelet stimuli.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Agent/Action</th>
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<tbody>
<tr>
<td>Acetylcholine*</td>
<td>Platelet-activating factor (Paf-acether)</td>
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<tr>
<td>ADP</td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td></td>
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<tr>
<td>Calcium ionophore</td>
<td></td>
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<tr>
<td>(A23187, ionomycin)</td>
<td></td>
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<tr>
<td>Catecholamines*</td>
<td>Latex particles</td>
</tr>
<tr>
<td>(Epinephrine,</td>
<td></td>
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<tr>
<td>norepinephrine)</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Polymer surfaces</td>
</tr>
<tr>
<td>Collagen</td>
<td>Prostaglandin endoperoxide and analogues*</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Shearforces</td>
</tr>
<tr>
<td>Elastase</td>
<td></td>
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<tr>
<td>Glutaraldehyde</td>
<td></td>
</tr>
<tr>
<td>H202</td>
<td></td>
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<tr>
<td>5-Hydroxytryptamine*</td>
<td>(N-ethylmaleimide, diamide, thimerosal)</td>
</tr>
<tr>
<td>Immune complexes</td>
<td></td>
</tr>
<tr>
<td>Kaolin</td>
<td></td>
</tr>
<tr>
<td>Low temperatures (ice-bath)</td>
<td></td>
</tr>
<tr>
<td>Methylmercury chloride</td>
<td></td>
</tr>
<tr>
<td>Tryptsin</td>
<td></td>
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<tr>
<td>Tumour-promoting phorbol</td>
<td></td>
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<tr>
<td>diesters (phorbol myristate acetate, phorbol dibutyrate)</td>
<td></td>
</tr>
<tr>
<td>Ultraviolet light</td>
<td></td>
</tr>
<tr>
<td>Unsaturated fatty acids*</td>
<td></td>
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<tr>
<td>Vasopressin</td>
<td></td>
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<tr>
<td>Vitamin A*</td>
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</table>

*Species-dependent variations are found in the response of platelets to these stimuli; **Reported for rabbit platelets.

responsiveness and previous history of the platelets, on the other. In addition, platelet activation is inherently heterogeneous in the sense that no two platelets in the same activated sample is in an identical state of activation at a given moment. This type of heterogeneity which seems to be an important aspect of organic life is likely to have important consequences for platelet aggregation and its regulation.

Aggregated platelets may dissociate from aggregates and regain their original shape but may become refractory to aggregation by the same agonist, again.

Biochemistry of platelet activation

The consensus view on the biochemical mechanisms of platelet activation may be summarized as in Figure 1.

Occupation of a sufficient number of surface receptors by an agonist triggers the activation of a guanine nucleotide-binding protein (G-protein) linked to phospholipase C (PLC) at the cytoplasmic side of the plasma membrane. The nature of this G-protein and whether the linkage is direct or indirect are matters which call for elucidation. Activated PLC catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) at resting intracellular calcium ion concentration (Ca2+) of 100 nM) to produce two key second messenger molecules—the hydrophilic inositol 1,4,5-trisphosphate (IP3) and the lipophilic diacylglycerol (DAG). IP3 is thought to release Ca2+ ions stored, apparently, in the dense tubular system (DTS) (see Figure 2) transiently raising

Ca2+ which may be regulated by cAMP levels and protein kinase C (PKC) activities which change during platelet activation. Receptor activation also opens the so-called receptor-operated calcium channels which in the presence of extracellular Ca2+ ions (~1 mM) add to the Ca2+ and help to sustain it longer, at ~1-1.5 μM.

Ca2+ has many direct and indirect effects on several key enzymes and proteins that control platelet activation.

Acting synergistically with DAG and in presence of membrane phospholipids Ca2+ activates PKC, leading to protein phosphorylation (mainly a 40-47-kDa protein is phosphorylated), and related changes in cell responses, mainly secretion, in this instance. But the molecular details are not yet clear. The secretions cause activation of platelets or augment the actions of other agonists. In addition to this positive effect, PKC seems to exert a negative control over Ca2+, cAMP levels and receptor-effector coupling.

Another postulated effect of Ca2+ is the activation of phospholipase A2 (PLA2), although Ca2+-independent activation of the enzyme can and does occur. Once activated PLA2 sets free another key biomolecule, namely, arachidonate, from phospholipids, mainly phosphatidylethanolamine. The mechanisms regulating arachidonate release are not understood at present.

Free arachidonic acid is quickly converted by the membrane-bound haem-iron enzyme, cyclo-oxygenase,
CO) to the unstable \( t_{1/2} = 5 \text{ min} \) products, prosta-
glandins G2 and H2 (PGG2 and PGH2). Only PGH2
accumulates and is converted by yet another haem-ion
enzyme, thromboxane synthetase, to thromboxane A2
(TXA2) which is even more unstable \( t_{1/2} = 0.5 \text{ min} \).
Both PGH2 and TXA2 are potent agonists for human
platelets acting on the same cell-surface receptor and
inducing platelet activation by way of the PLC–PKC
pathway. The mechanism by which unstable molecules
generated at the cytoplasmic side of the plasma
membrane are transported to the cell surface is
unexplained.

The enzyme cyclo-oxygenase is irreversibly inactiva-
ted by aspirin which explains some but not all of its
platelet antagonistic effects.

A smaller proportion of liberated arachidonate is
acted on by lipoxygenases (LO) which are nonhaem-
iron enzymes, producing hydroperoxy- and hydroxye-
oxo acids whose biological roles are only just
beginning to be understood.

Platelets as a model system of intercellular
interactions

Platelets can be isolated, in defined media, employing
only gentle methods, and they can be preserved in a
near-native state for at least an hour which is long
compared to their activation time, a few tens of seconds
or, at the most, a few minutes. And their activation is
unhampered by nucleic acid and protein synthesis and
a valuable collection of authoritative articles on platelet
methodology has become available recently\(^{18}\).

Specific cell–cell recognition reactions, of which
platelet aggregation is an example, are an intricate
problem in modern cellular biology and biochemistry
we are only just beginning to understand\(^{19–21}\).

The mechanism essentially involves activation-
dependent interaction of cell-adhesion receptors and
their counter-receptors on apposite cells\(^{19,20}\). Cell-
adhesion receptors belong to various families and
subfamilies\(^{19,20}\). Glycoprotein (GP) IIb–IIIa, the puta-
tive cell-adhesion receptor of platelets, is a platelet-
specific member of the integrin family\(^{19–21}\).

Cell-adhesion receptor–counter-receptor interactions
differ from simple ligand–receptor interactions in that
they require metabolic energy-consuming\(^{22,23}\) cell-
surface modification steps in addition to cell movement
and, relatively long, time\(^ {24}\). Very little is known about
the nature and regulation of these steps. Platelets,
because of their easy accessibility and rapidity of
responses, offer rare opportunities for investigating such
problems. The present article is an attempt to present

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Figure 2. Discord platelet. a. The lentiform shape of blood platelets is well preserved in samples fixed in glutaraldehyde and critical
point dried for study in the scanning electron microscope. The indentation (arrow) apparent on the otherwise smooth surface of the
platelet indicates sites where channels of the surface-connected open canicular system (OCS) communicate with the cell exterior
(\( \times 25,000 \)). Re-tung bovine platelets do not have OCS. b. The cell in this sample has been sectioned in the equatorial plane. A
circumferential band of microtubules (MT) supports the discoid configuration. Numerous granules G (alpha granules), a few
mitochondria M and occasional electron dense bodies DB (dense granules) are randomly dispersed in the cytoplasmic matrix.
Glycogen Gly is concentrated in masses or occurs as single particles. Clear channels of OCS follow tortuous courses through
the cytoplasm. Elements of the dense tubular system DTS of channels are often associated with the circumferential bundle of
microtubules. Interaction between the two channel systems results in the formation of a twisted mass of membranous elements
referred to as the membrane complex MC (\( \times 28,000 \)).

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platelet aggregation with this new perspective in mind and in relation to work in my laboratory. As a prelude to this a brief discussion on the basic responses of platelets in relation to their aggregation is given. Reference to original work may be found in the reviews referred to here.

Shape change

Off duty, platelets are maintained in a characteristic discoid shape by an elaborate system of dynamic structural elements but mainly by a circumferential band of microtubules (Figure 2). Discoid platelets are nonadhesive and nonaggregable. Their plasma membrane bears an outer coat or glycocalyx (span ~ 20 nm) which is retained during platelet activation and aggregation but the composition of the plasma membrane changes.

The plasma membrane is the repository of specific receptors for many platelet stimuli and the site of the integrin GPIIb-IIIa regarded as being important for the adhesive and aggregatory reactions of platelets. The Ca$^{2+}$ ion complex of this glycoprotein functions as a receptor for the adhesive glycoproteins fibrinogen, fibronectin and von Willebrand factor, both in plasma and platelet granules. The amino-acid sequence Arg–Gly–Asp (RGD in single-letter amino acid code) present in them seems to be important for their recognition. The integrin is evenly distributed in resting discoid platelets in a conformation not available for plasma fibrinogen binding but becomes available after activation.

Within a few seconds of activation (t$_{1/2}$ ~ 4 sec under unstimulated conditions) platelets lose their discoid shape and become spheroidal having increased permeability to divalent cations. Subsequently they extrude thin pseudopods and other surface protrusions and pseudopods are important for platelet aggregation. Pseudopod formation without spheration induced by epinephrine and tumour-promoter phorbol diesters may then be regarded as shape changes, although the term usually refers to the disk-to-spiny sphere transformation.

It is now widely believed that an early and essential common event in agonist-induced platelet activation is an elevation of Ca$^{2+}$ but the precise time of its occurrence, its source and mode of occurrence, and its specific role in platelet shape change have not yet been clearly defined. Ca$^{2+}$ ion-calmodulin-dependent myosin light chain phosphorylation may be involved.

Shape change may require the polymerization of submembranous actin, changes in the components of the membrane skeleton, constriction and breakdown of microtubule or actin–myosin interactions and cytoskeletal structures. Extrusion of pseudopods requires the formation of new actin filaments. Arrhenius activation energy (Ea) for shape-change is ~ 15.6 kcal mol$^{-1}$ (ref. 11).

Secretion

Different numbers of four types of granules (Table 2) which may have subtypes, are found dispersed randomly in the cytoplasm of the resting cells. These organelles move towards the cell centre with tight rings of microtubule coils (see Figure 4) in preparation for the secretion of their contents which is correlated with (i) transformation of the aggregatory reaction from reversible to irreversible type and (ii) formation of prostaglandin endoperoxides and thromboxane A2. As mentioned earlier Ca$^{2+}$ and PKC are believed to be

<table>
<thead>
<tr>
<th>Table 2. Platelet storage granules and their contents.</th>
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<tbody>
<tr>
<td><strong>Granule</strong></td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Alpha granules</td>
</tr>
<tr>
<td>Dense granules</td>
</tr>
<tr>
<td>Lysosomes</td>
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<tr>
<td>Peroxisomes</td>
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involved in the secretory reactions whose molecular details are not clear.

**Aggregation**

Adequate concentrations of an aggregating agent at appropriate pH and temperature induce aggregation of metabolically active viable platelets. The optimum pH is in the 7.4 to 8.0 range and little aggregation occurs below pH 6.4 and above pH 10. Optimum temperature is in the 35-40°C range and inhibition occurs above 40°C. Ea for platelet aggregation (under conditions of flow-induced shear) is 16 kcal mol⁻¹ (ref. 43). This value is not much different from the Ea for agonist-induced shape change reactions of unstimulated platelets. But the values may not be strictly comparable because agita-
tion of platelets aerobically is thermogenic in the microcalorimeter. The molecular basis of this heat production is not clear but probably involves sulphydryl oxidation.

We have found an Ea of 20 ± 1 kcal mol⁻¹ (mean ± SE, n = 3) for the microaggregation of gel-filtered calf platelets by ADP, thrombin and H202, showing that there is no agonist-specific difference in activation energy. Aggregation per se produces little heat effects, suggesting that the driving force may be entropic, probably hydrophobic, in origin because aggregation occurs in presence of salt and so contributions from electrostatic forces may be neglected.

Basal heat production by platelets has an Ea of 12.4 kcal mol⁻¹ (ref. 46). The additional Ea required for shape change and aggregation over and above that needed for basal heat production is not much larger than that which is necessary for a membrane-diffusion process, a common requirement for specific cell-cell recognition reactions.

Platelets have a negatively charged glycoalyx. Glycoalyces, in general, serve as barriers to close cell-cell contact by their repulsive charge interactions and the loss of entropy involved in compressing or interdigitation of the glycoalyces of contacting cells. Platelet–platelet contact in aggregates occurs without apparent interdigitation or compression of their glycoalyces so that a distance of ~40 nm is found between plasma membranes of contacting platelets in aggregates (Figure 4). This sort of distance is commonly found in integrin-mediated cell–cell interactions which allow lateral flow of membrane lipids and proteins. This may help the dynamics of extrusion of pseudopods which have different membrane lipid and protein composition compared to main body. The importance of pseudopods for platelet aggregation has been repeatedly stressed by different workers.

Activated platelets contact each other with rapidly forming pseudopods aggregation depending on the number and length of pseudopods and the availability of GPIIb-IIIa in them. Further, 85-90% of all platelet contacts in aggregates is pseudopod–pseudopod or pseudopod–main body type. Pseudopods of the right form and membrane composition are required because formation of short blunted projections which sequester GPIIb-IIIa is inhibitory. So extrusion of pseudopods which sequester GPIIb-IIIa its counter-receptor or other components required for aggregation could form a mechanism of platelet malfunction and the action of certain antiplatelet drugs. Interestingly EDTA, prostaglandin I2, prostaglan-
din E1, chlorpromazine and lysophosphatidyl cholines all induce extrusion of pseudopods. Intriguingly prostaglandin E1 aggregates rabbit platelets.

**Requirement for fibrinogen.** Activated platelets avidly bind fibrinogen in the presence of Ca²⁺ ions and their addition is required for washed human platelet aggregation. But fibrinogen requirement is not attested by all investigations (ref. 56 and references cited therein) and evidence showing that fibrinogen is not essential or is not solely responsible for holding platelets in primary aggregation and that fibrinogen comes into the picture only after platelet–platelet contacts have already been made has been published recently. The significance of fibrinogen binding to activated platelets, other than in aggregation, has also been discussed.

**Requirement for stirring.** The notion that a ‘collision force’ of stirring is necessary to induce aggregation of activated platelets is pervasive in the platelet literature.
but is contradicted not only by thermodynamic data which suggest that platelet activation leads spontaneously to aggregation but also by experimental demonstration of agonist-induced aggregation of platelets of various species by Brownian motion\textsuperscript{36,60–64}. A convenient method of following this sort of aggregation in a spectrophotometer was devised in my laboratory\textsuperscript{62–64}. This method measures single-platelet recruitment into aggregates (predominantly dimers)\textsuperscript{64} (Figure 5) in contrast to the conventional photometric method and the whole blood aggregometer, which measure clumping of primary aggregates\textsuperscript{41}. These latter methods are therefore unsuitable for studying the initial stages of platelet aggregation.

Although stirring is not essential for agonist-induced aggregation of platelets, stirring platelets is thermodic and does modify platelet aggregation reactions both at low\textsuperscript{62} and at high\textsuperscript{36} platelet concentrations. Stirring reduces the induction time for aggregation\textsuperscript{36,62} and sustains aggregation rates longer\textsuperscript{62}. But it seems to have an undesirable effect of obliterating genuine differences in the aggregatory properties of platelets\textsuperscript{65}. So it is best to avoid stirring when looking for small differences in aggregation rates.

Kinetics

As already noted platelet aggregation and extrusion of pseudopods are causally connected. Extrusion of pseudopods is an agonist-induced stochastic process\textsuperscript{14} involving two consecutive reactions, namely, disk-to-sphere transformation and pseudopod formation, each having the same rate constant \(k = 0.16\text{ s}^{-1}\) under nonstir conditions\textsuperscript{10,14}. Each of these first-order processes, therefore, has a half-life \(t_{1/2} = \ln 2/k\) of \(\sim 4\) sec. So a lag time of at least this duration may be expected before aggregation becomes significant provided that only unactivated platelets are present initially and stirring is not resorted to. This, in fact, is found (Figure 5) (see also ref. 64). The lag time is independent of platelet concentration (unpublished results) but increases with a decrease of either agonist concentration\textsuperscript{64} or temperature from the optimum of 36–38°C (unpublished).

The 'stickiness' of platelets increases with agonist concentration\textsuperscript{61}. Since it also depends on the number and length of pseudopods extrusion of pseudopods and their lengthening may be slower at lower agonist concentrations. But this has not been verified. More or longer pseudopods are formed\textsuperscript{11} at 37°C than at 31°C which might explain the decrease of lag time with increase of temperature. Stirring also decreases lag time\textsuperscript{62}.

The occurrence of a lag time inversely related to agonist concentration but independent of platelet concentration is predicted by the sequential shape change and interaction model of aggregation\textsuperscript{41}. The mathematics remains to be worked out. Although the lag time varies quite considerably with agonist concentration\textsuperscript{64} the platelets are not desensitized in the lag period but only after aggregation–disaggregation reactions have occurred (ref. 62 and unpublished results).

Traditionally, platelet aggregation is regarded as being analogous to colloidal particle aggregation and it is believed that the Smoluchowski theory\textsuperscript{66} of diffusion-controlled, rapid, irreversible aggregation of spherical particles is applicable. But experimental data do not support this belief\textsuperscript{61,67,68}. The inset of Figure 5 shows a plot of platelet aggregation kinetic data according to a prediction\textsuperscript{60,61} of the Smoluchowski theory. A linear relationship is expected but not found. If a second-order rate constant is calculated, assuming the data to be linear, the value turns out to be larger than even the theoretical upper limit of bimolecular reactions in solution. The same situation is found with data published in the literature\textsuperscript{60,61}. Deviations found in colloidal systems are in the opposite direction. This as well as other considerations\textsuperscript{69} make it appear that platelet aggregation is a first-order reaction as predicted by the sequential shape change and interaction model.

![Figure 5. Time-course of single-platelet recruitment into aggregates (□) and the formation of dimeric (●) and trimeric (▲) species of aggregates expressed as per cent of single platelets remaining. In the inset, the reciprocals of the concentration of single platelets remaining, derived from the smooth curve drawn through the experimental points, were plotted against the corresponding t. Second-order rate constant calculated from the data assuming a linear relationship, was \(1.2 \times 10^{-9}\text{ cm}^3\text{ platelets/sec. Platelet concentrations were determined microscopically as in ref. 64.}\)
of Jamaluddin and Krishnan. But there are certain aspects of platelet aggregation that set limits to time of applicability of theoretical models. Platelet aggregation is a dynamic process which proceeds by reversible equilibria in the initial stages, and platelets which have dissociated from aggregates may become desensitized, by an as yet unknown mechanism, towards their subsequent aggregation. This, together with the occurrence of secretion and the formation of significant proportions of larger aggregates (larger than dimers) at later times, can complicate the time-course of aggregation. For these reasons aggregation kinetics can and does become complex after about 60 sec and stirring will add to the problem. It is important therefore to study the aggregation profile as early as possible after initiation of aggregation, under unstirred conditions. The spectrophotometric method of Jamaluddin and Krishnan is ideally suited for this purpose. The results of its application to the kinetics of aggregation of gel-filtered calf platelets by thrombin, and a combination of thrombin and ADP simultaneously, are shown in Figure 6. The apparently positively cooperative pattern or maximum rate of aggregation induced by thrombin is not changed in the presence of ADP but the S0.5 of thrombin is reduced. A synergistic effect is found at nonsaturating concentrations of thrombin. Additional data have indicated that ADP reduces the lag time for aggregation found with thrombin alone without modifying the Ea, showing that no new activation pathways are involved in the synergistic action of the two agonists. Synergism of agonist pairs is a dark area in platelet research.

Cooperativity of platelet aggregation kinetics was discovered with the application of the spectrophotometric method and appears to be an important mechanism of modulating platelet aggregation. Together with the sequential shape change and interaction model of aggregation it may also serve to gain insights into the action of antagonist pairs.

REVIEW ARTICLES

41. Zucker, M. B., in ref. 18, pp. 117–133.

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