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Topical Review

Membrane Phospholipid Asymmetry and Signal Transduction

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Introduction

It is now 75 years ago that the lipid bilayer concept was proposed by Gorter and Grendel (1925). Since then many concepts about the organization of lipids and proteins in the membrane have been proposed, including the milestone of the "fluid mosaic bilayer concept" of Singer and Nicolson (1972). In this concept (glyco)proteins can laterally diffuse within the fluid (glyco)lipid bilayer. This concept is appealing in its simplicity, but it is now generally accepted that there can be many domains of proteins within the fluid bilayer. These include, interaction with cytoskeletal proteins, such as coated vesicles, or in cellular functionalities such as gap junctions and desmosomes, or in the well-known crystalline domain of the purple membrane or under conditions where lipid phase transitions by solidification of lipids introduce protein domains in the membrane (*see* for review Verkleij, 1989). In the recent years it has been suggested that in rafts and/or caveolae, which are enriched in sphingomyelin, cholesterol and glycolipid, the lipids induce domains in which specific proteins are present (Pralle et al., 2000). These proteins fit in these domains because they match with their α-helices within the thickness of the lipid bilayer in these domains (for reviews *see* Killian, 1998; Dumas et al., 1999).

Besides domains within the plane of the membrane, differences in the two membrane halves exist, the so-called lipid asymmetry. This asymmetry and its role in cellular (dys)functioning and signal transduction is the topic of this review.

Phospholipid Asymmetry

The phospholipid classes in a biological membrane are not randomly distributed over the two membrane halves and the concept of phospholipid asymmetry has first been postulated by Bretscher (1972). Bretscher and also Gordesky and Marinetti (1973) based their concept of an asymmetrical transbilayer lipid distribution on experiments using chemical probes, which indicated that the amino-containing phospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), reside predominantly in the cytoplasmic leaflet of the plasma membrane. Using phospholipase A_2 (PLA_2) and sphingomyelinase C (Sphase) to hydrolyze the phospholipids present in the outer monolayer, the complete transbilayer distribution of the phospholipids in the erythrocyte membrane was elucidated (Verkleij et al., 1973). The clue of those experiments, which elucidated the asymmetric distribution, was the sequential treatment of the erythrocyte with PLA_2 (*Naja naja*) and Sphase C (*S. aureus*), thus preventing any lysis of the cells to occur, despite the essentially complete hydrolysis of the phospholipids in the outer monolayer. Indeed, 48% of the total phospholipids was hydrolyzed, comprising the major fraction of the choline containing phospholipids, phosphatidylcholine (PC) and sphingomyelin (Sph), whereas only a minor fraction of phosphatidylethanolamine (PE) was degraded. No degradation of the negatively charged phosphatidylserine (PS) and phosphatidylinositol (PI) was detected. The actual transbilayer distribution of the phospholipids of the erythrocyte membrane is shown in Fig. 1. This figure also shows the average composition of the

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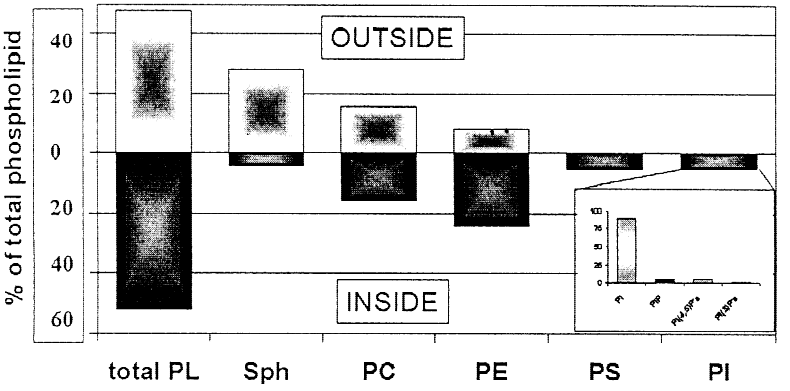

Fig. 1. This figure shows the asymmetrical distribution of the phospholipids of the erythrocyte membrane. The insert shows the composition of the different PI's according to the phosphorylation of the inositol group.

different PIs, with regard to the phosphorylation of its inositol ring, present in the cytoplasmic leaflet (as discussed by Rameh and Cantley (1999)). The specific role of these different PIs will be discussed below. Membrane splitting analysis by Fisher confirmed the asymmetrical distribution of PC. Moreover it showed that the outer leaflet is enriched with cholesterol (Fisher, 1976). Comparable transbilayer distribution of the phospholipid classes has also been demonstrated for the platelet (Chap, Zwaal & van Deenen, 1977) the cardiac myocyte (Post et al., 1988) and other cell types, as reviewed in Op den Kamp, 1979; Zachowski 1993, Deveaux 1992, Roelofsen & Op den Kamp, 1994. The concept of lipid asymmetry is nowadays widely accepted as a general membrane phenomenon.

The maintenance of the asymmetrical phospholipid distribution is believed to be attributable to two processes: (i) an ATP dependent translocation of the aminophospholipids towards to cytoplasmic leaflet, as originally described by Seigneuret and Deveaux (1984), and (ii) an interaction between the membrane cytoskeleton and the head groups of the aminophospholipids, as suggested first by Haest et al. (1978). As reviewed by Roelofsen and Op den Kamp (1994) a loss of phospholipid asymmetry only occurs when both of these processes are malfunctioning.

Lipid asymmetry is not restricted to the polar head group of the phospholipids, but also includes asymmetry of the hydrophobic parts. Exemplified, it is found that the phospholipids in the inner leaflet of the plasma membrane of myocardial cells are enriched in plasmalogens and in unsaturated fatty acids, such as arachidonic acid (Post et al., 1988*b*; Matos et al., 1990). The latter makes sense in the light of being the substrate of cytosolic phospholipase A_2, *see* below.

A consequence of the above described lipid asymmetry is the bilayer couple theory, proposed by Sheetz and Singer in 1974. They realized that the two halves of a membrane respond differently to a perturbation, resulting in alterations in the curvature of the membrane. Insertion of additional hydrophobic molecules into the outer leaflet of a membrane leads to evagination, whereas insertion into the inner leaflet causes invagination. This has been exemplified with the erythrocyte where expansion of the outer leaflet results in creation and expansion of the inner leaflet leads to stomatocyte formation.

Loss of Membrane Phospholipid Asymmetry

A rapid collapse of the phospholipid asymmetry in the plasma membrane of blood platelets is observed upon activation of the cells, resulting in an exposure of PE and PS (as reviewed by Schroit and Zwaal (1991)). Other conditions leading to a loss of asymmetry are: aging of erythrocytes (Diaz & Schroit, 1996), sickle cell anemia (Franck et al., 1985), maturation of sperm cells (Nolan et al., 1995), the apoptotic process (Fadok et al., 1992) and metabolic inhibition or simulated ischemia of myocardial cells (Post, Clague & Langer, 1993, Musters et al., 1993). Exposure of PS at the surface of cells can activate processes such as blood coagulation (Schroit & Zwaal, 1991) and macrophage recognition and subsequent fagocytosis (Fadok et al., 1992). As will be discussed later, the presence of PS in the cytoplasmic leaflet plays a crucial role in excitation contraction coupling and activation of enzymes.

With regard to the loss of the asymmetry in myocardial cells, it is of interest to note that at the plasma membrane only a loss of the asymmetrical distribution of PE is observed (Post et al., 1993; Musters et al., 1993), before cell lysis occurs. The outward flow of PE, although reversible provided that energy supply is restored in time (Musters et al., 1996), seems to be involved in subsequent cell death. This might be due to the fact that the outward movement of PE is not compensated for by an influx of other membrane components, which results in an increased pressure in the extracellular leaflet. This will, according to the bilayer couple theory, lead to evagination of the membrane, which is indeed found by electron microscopy (Musters et al., 1991). Recent ex-

periments showed that compensation of this increase in pressure in the outer monolayer attenuates evagination and cell lysis during simulated ischemia (Steenbergen et al., *in preparation*). These evaginations may induce shedding off of vesicles by the cells and upon shedding of these vesicles also PS asymmetry is lost, resulting in an exposure of PS (M.C.A. Stuart and J.A. Post, *unpublished results*). Also during apoptosis and blood platelet activation shedding of vesicles occurs accompanied with a subsequent exposure of PS, which is shown using annexin-gold labeling at the electron microscopic level (Stuart et al., 1995,1998). Using this approach no initial PS exposure is seen at the plasma membrane itself and thus it is of interest to speculate that the initial loss of PS asymmetry is limited to vesicles which have lost their functional relation with the cell, although maybe still attached. This means that ATP levels cannot be maintained and the interaction of the cytoskeleton with the membrane lipid is decreased.

When ischemia is prolonged and/or is followed by reperfusion, the increase in cytosolic calcium leads to a destruction of the plasma membrane, in which several processes are involved, such as phospholipid phase separation, expression of nonbilayer behavior of PE, generation of reactive oxygen species and activation of phospholipase A_2 (Musters et al., 1993; Steenbergen et al., 1997). This important role of PE is clearly illustrated by the fact that lowering the PE content indeed diminished cell death during simulated ischemia and reperfusion (Post, Bijvelt & Verkleij, 1995).

Phospholipid Asymmetry and Excitation Contraction Coupling in the Heart

The negatively charged phospholipids present in the cytoplasmic leaflet could serve as ion binding sites and as such might serve as a storage site for Ca^{2+}. Experimental data and mathematical modeling, as discussed below, suggest an important role for negatively charged phospholipids, in the cytoplasmic leaflet of the plasma membrane of myocardial cells, in the process excitation-contraction coupling of these cells. Functional studies on calcium compartmentation in single myocardial cells revealed a very rapid exchangeable pool of calcium, with a $t_{1/2}$ of less than 1 sec and the rapidity of this exchange placed this calcium pool at or near the plasma membrane of the myocardial cell, the sarcolemma (Kuwata & Langer, 1989). Comparison of the amount of Ca^{2+} bound to isolated sarcolemmal vesicles and to lipidic vesicles made out of lipid extracts of these vesicles revealed that up to 80% of the sarcolemmal bound calcium could be accounted for by the sarcolemmal phospholipids (Philipson, Bers & Nishimoto, 1980). Of the two classes of sarcolemmal Ca^{2+}-binding sites the low affinity, high capacity one (K_d 1.1 mM, 84 nmol/mg) was not affected by neuraminidase or protease treatment and was lost upon phospholipase C treatment or lipid extraction (Post & Langer, 1992), again indicating the sarcolemmal phospholipids as the molecular sites for the sarcolemmal Ca^{2+} binding sites. The earlier described asymmetrical distribution of the sarcolemmal phospholipids places those phospholipids representing the calcium binding sites at the cytoplasmic leaflet. However, it is to be noted that the maximal cytosolic calcium concentration in a myocardial cell at rest is in the range of 100–200 nM and during contraction will not exceed 2 μM, and thus very little calcium will be bound to these low affinity binding sites. This, unless they would be present in a subsarcolemmal microenvironment with a much higher Ca^{2+} concentration than the average cytoplasmic concentration. A candidate for this microenvironment is the so called diadic cleft, a 12–15 nm space between the junctional sarcoplasmic reticulum (JSR) membrane and the inner leaflet of the transverse (T) tubular membrane (Langer & Peskoff, 1996). It is into this cleft that calcium is released from its sarcotubular storage site to initiate contraction.

On the basis of the cleft structure, Ca^{2+} channel and the Na/Ca^{2+} exchange function and the inner sarcolemmal membrane characteristics a model for Ca^{2+} concentration and movements has been constructed (Post et al., 1992; Langer & Peskoff, 1996; Peskoff & Langer, 1998). The model indicates that calcium is released upon excitation from the SR and would result in a local calcium concentration above 1 mM in the cleft. In the absence of the subsarcolemmal phospholipid calcium binding sites the calcium concentration falls rapidly (<3 msec) to control levels once release from the SR has ceased. Insertion of the lipidic Ca^{2+} binding sites in the model results in a somewhat lower calcium concentration (600 μM), due to the buffering capacity of the sites. More strikingly, upon cessation of the release from the JSR calcium concentration in the cleft remains above 20 μM for up to 200 msec.

Does this have any physiological implications? The major system responsible for removing Ca^{2+} from the myocardial cell, the Na/Ca^{2+} exchanger, is preferentially localized to that part of the sarcolemma where also the above-mentioned diadic clefts are present (Frank et al., 1992). Reported values for the $K_{d,Ca}$ for activation of this exchanger vary between 6 and 20 μM (Nicoll et al., 1991; Hilgemann et al., 1992) and thus, were the exchanger to face the average cytoplasmic Ca^{2+} concentration, little calcium would be removed from the cell during the cardiac cycle and calcium overload would be the final consequence. The increased Ca^{2+} concentration, as modeled in the dyadic cleft, exposes the exchanger to a Ca^{2+} concentration well above its K_d for a relatively prolonged period, thus allowing optimal operation of the Na/Ca^{2+} exchangers and removal of amounts of Ca^{2+} from the cell sufficient to maintain steady-state intracellular levels of

calcium. In this way, the negatively charged phospholipids, present in the cytoplasmic leaflet, would play a crucial role in maintenance of Ca^{2+} homeostasis during excitation-contraction coupling. It might also explain the observed stimulation of both the Na/Ca^{2+} exchanger and Ca^{2+}-ATPases by anionic lipids (Roelofsen & van Deenen, 1973; Niggli et al., 1984; Vemuri & Philipson, 1989). Also PI products have been shown to affect the Na/Ca^{2+} exchanger activity (He et al., 2000).

Phospholipid Asymmetry and Receptor-Mediated Signal Transduction

Signal transduction via membrane receptors starts with binding of the ligands to their receptors which results in activation of the receptors. In the case of tyrosine kinase receptors (TKR) this occurs by dimerization or oligomerization of the receptors which lead to auto/cross phosphorylation of the TKR (Heldin et al.,1995), whereas in case of the seven α helix receptor binding of the ligand with its receptor leads to a conformational change of the cytosolic loops of the receptor, which leads to binding and activation of G-proteins by an exchange of GDP by GTP (*see* Berridge, 1993).

Specific binding motifs in the activated TRK receptor, containing a phosphorylated tyrosine as a basic motive, bind their substrates through src homology 2 (SH2) domains (*see* for review Cohen, Ren & Baltimore, 1995). Examples of these substrates are PI 3-kinase, PLCγ, c-Src, GAP and the adaptor proteins Grb2 and Shc. In case of the seven α-helix receptors PLC$_\beta$, and PI 3-kinase are bound to and activated by G-proteins. Activation of the docked enzymes occurs by phosphorylation in case of the tyrosine kinase receptor substrates or by the binding to activated G-proteins. In the next pages the involvement of the phospholipids classes present in the cytoplasmic leaflet of the plasma membrane and involved in transduction of the signal will be discussed. We will limit this discussion to concepts on the topology and temporal aspects of the phospholipids present, or generated, in the cytoplasmic leaflet of the membrane, since extensive reviews have been written on the role of lipid second messengers in activating signal transduction cascades (Nishizuka, 1992; Berridge, 1993; Liscovitch & Cantley, 1994; Rameh & Cantley, 1999).

The Role of Phosphorylated PI Products

The classical role for phosphorylated PI products is attributed to PI (4,5) P_2, which is the substrate for PLCγ or β in the tyrosine kinase and G-protein-coupled receptor pathway respectively (Berridge, 1993). PI (4,5) P_2 hydrolysis by PLC starts within seconds upon receptor activation producing diacylglycerol (DAG) and IP_3. Next to this, PI (4,5) P_2 is also shown to play an important role in membrane localization of molecules, including signal transduction molecules by their ability to bind proteins containing a pleckstrin homology domain (PH) specific for PI (4,5) P_2 (Lemmon et al., 1996, 1997). PI (4,5) P_2 is constitutively present, exclusively at the cytosolic leaflet in the plasma membrane and comprises about 5% of total phosphoinositides.

In contrast to this, the products of PI 3-kinase are present only in trace amount and upon activation of the tyrosine kinase receptor class I PI 3-kinase is activated and PI-3 products are generated. PI (3,4,5) is proposed to bind PLCγ2 in vitro by its PH domain, which can be inhibited by the PI 3-kinase inhibitor wortmannin (Gratacap et al., 1998) and PLCγ has been found to bind with its PH domains to PI (3,4,5) P_3, which mediates PLCγ translocation to the plasma membrane in response to growth factors (Falasca et al., 1998). PI (3,4) P_2 can bind to other proteins, such as PKB, which have PH domains more specific for these PI3 kinase products (Andjelkovic et al., 1997; Franke et al., 1997). In vitro experiments have shown that PI (3,4) P_2 activates PKB, whereas PI (3,4,5) P_3 slightly inhibits PKB (*see* review Rameh & Cantley, 1999). The suggestion that PKB can be activated directly by PI (3,4) P_2 is based on the observation that binding of PI (3,4) P_2 to the PH domain of PKB in vitro causes activation of the kinase (Frech et al., 1997). PKB is activated in any case by phosphoinositide-dependent kinase 1 (PDK1) (Alessi et al., 1998), a constitutively active kinase that becomes localized at the membrane by binding to PI (3,4,5) P_3 with its PH domain, in response to receptor activation (Anderson et al., 1998). Recently it has been reported that PDK1 is maximally activated by sphingosine, a breakdown product of spingomyeline and ceramide (King et al., 2000). Upon receptor activation PI (3,4,5) P_3 is increased first after 30 seconds and is likely dephosphorylated subsequently by phosphatase 2A to PI (3,4) P_2 at the moment that PKB becomes activated (Franke et al., 1997).

It is relevant to speculate where and when these interesting phosphorylated PI products are present and generated. PI (4,5) P_2, the substrate of PLC, is constitutively present in the cytoplasmic leaflet and most likely homogeneously distributed. Moreover, it is supposed to be bound with pleckstrin homology (PH) domain containing proteins, like pleckstrin, spectrin, but also PLCδ. Pleckstrin and spectrin bind with low affinity (K_d > 30 μM (Harlan et al., 1994). However, they can be present at high concentrations since they also bind via protein-protein interaction with transmembrane proteins, for instance spectrin binds to band 3 in the case of the erythrocyte (Branton, Cohen & Tayler, 1981) and thereby the membrane skeleton can interact with PIs and other negatively charged phospholipids (Isenberg & Niggli, 1998).

What about the PI 3-kinase products? In the resting

state only trace amounts of these molecules are present. Since the enzymes that are necessary for their biosynthesis are activated near the receptor, immediately upon receptor activation, it is reasonable to assume that PI (3,4) P_2 and PI (3,4,5) P_3 are synthesized near the activated receptor and form a dynamic lipid microdomain, exclusively in the cytoplasmic leaflet of the membrane for a limited amount of time (sec-min). As discussed above, these products serve as docking sites for signal transduction molecules as PLCγ and PKB, which have a preferential interaction with PI (3,4,5) P_3, and PI (3,4) P_2, respectively. Of importance are the concentrations and the affinity of the PH containing proteins which bind to the polyphosphoinositides. The question is whether the concentration of these molecules, for instance PLCδ, which has a relatively high affinity ($K_d = 1.7$ μM) for PI products (Lemmon et al., 1995), is high enough to result in binding. For that, the polymerization of actin-cytoskeleton at the site of activated receptors is of relevance. PLCγ, PI 3-kinase, PI 4-kinase and PI 5-kinase and many others can bind indirectly with the actin-cytoskeleton and thereby increase in concentration at those sites. Upon activation of membrane receptors actin polymerization is found at the site of the receptor activation, including membrane ruffles, with activated EGF receptor, and focal adhesions with activated integrines. Most of the receptors can bind actin either directly, such as EGF-receptor (Den Hartigh et al., 1992) or indirectly, for example via talin in case of the integrin receptor (Jockush et al., 1995). This polymerized actin, as visualized by immunofluorescence using phallocidin, appears within seconds upon activation of the receptor and disappears after about 10 min from the activated region of the membrane. Colocalization is found for the activated receptor, actin and PLCγ with immunofluorescence (Diakonova et al., 1992). In fibroblasts, immunofluorescence microscopy showed that endogenous Akt/PKB localized to membrane ruffles at the outer edge of the cell following mitogen stimulation, as did the PH domain of PKB, fused to GFP protein (Watton & Downward, 1999). Similar results were obtained in our laboratory (Doornbos, 2000). Biochemical fractionation showed that the activity of the PI-cycle enzymes, PI 3-kinase, PI 4-kinase, DAG-kinase, and PLC are associated with actin filament and that their activity is 2–3 times increased upon EGF receptor activation (Payrastre et al., 1991). PI 4-kinase and PIP 5-kinase, which are also involved in the PI metabolism, are also activated by the activated EGF receptor (Payrastre et al., 1990).

What is then the role of actin there during receptor activation? First of all as discussed above, it could be involved in the recruitment of signal transduction enzymes, which bind to the actin filament system, to the membrane area with activated receptors, where they bind to the receptors with their SH2 domains. Secondly, by the same mechanism actin may locally and temporarily stabilize the active signal transduction membrane complex, in order to enhance the signal of the lipid second messenger, which is necessary for binding and activation of PKC and PKB. In that respect it is of interest to mention that the destruction of the actin filament system by cytochalasin B inhibits the translocation of PKC to the membrane and subsequent desensitization of the EGF receptors (Rijken et al., 1998).

In conclusion both PLC and PI 3-kinase are recruited to the membrane by docking with phosphorylated tyrosines at the receptor by their SH2 domains. This in concert with actin polymerization to which these proteins are bound. Subsequently, PH domain containing signal transduction molecules can bind to their specific polyphosphoinositides in the inner leaflet of the membrane (Fig. 2).

The Role of Ceramides

The degradation of sphingomyeline, which is present in the cytoplasmic leaflet to a small extent, leads to formation of ceramides. Ceramides affect various cellular responses (Hannun, 1996) and recently it has been found that the production of ceramides in the inner leaflet may negatively regulate the activity of PKB by activating a phosphatase which dephosphorylate PKB on Ser 473 (Schubert, Scheid & Duronio, 2000). Furthermore, ceramides induce phosphorylation of PKCζ in cells and activates the enzyme in vitro (Muller et al., 1994). Doornbos found that PKB is negatively regulated by PKCζ (Doornbos et al., 1998) and recently Doornbos and van Bergen en Henegouwen in our laboratory found that ceramides induces inhibition of PKB, which is mediated by PKCζ (Doornbos, 2000). In Fig. 2, a schematic representation of a membrane ruffle containing activated receptors, where PI products and ceramides are increased to regulate PLCγ and PKB, as examples of signaling molecules activated at the cytoplasmic leaflet in microdomains, stabilized by actin skeleton, near the receptor.

The Ca Lipid Binding domains

Next to the role of phosphorylated PI products in the cytoplasmic leaflet of the membrane other lipids are involved both in binding and activation of signal transduction enzymes. The most classical is the activation of PKC. Most of the PKCs are activated upon the formation of diacylglycerol, generated in the cytoplasmic leaflet upon activation of PLC. Most of the PKC isoforms also need PS, constitutively present in the cytoplasmic leaflet, and calcium for their activation (Nishizuka, 1992). This activation also involves the formation of microdomains in the cytoplasmic leaflet containing PS (Yang & Glaser, 1996).

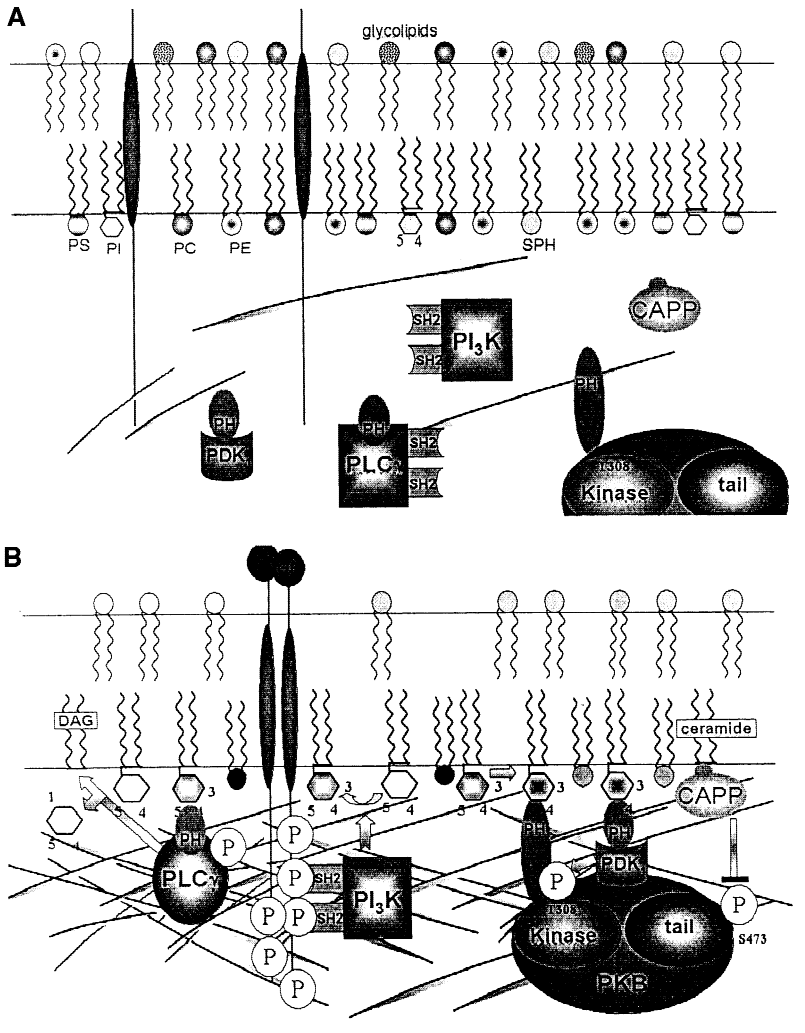

Fig. 2. Panel *A* shows the organization of the membrane at rest. Panel *B* shows that upon ligand binding receptors dimerize and cross-phosphorylate and the actin-cytoskeleton is increased. PI 3-Kinase is recruited to the plasma membrane and PI (3,4) P_2 and PI (3,4,5) P_3 are generated, which serve as binding sites for PKB, PDK1 and PLCγ, via their PH domains. PLCγ leads to the formation of DAG and IP_3, which both are involved in the activation of PKC. PKB, activated by the PI 3 kinase pathway via PDK1, can be inactivated by the production of ceramides. This activates a phosphatase (CAPP), that dephosphorylates PKB, and activates PKCζ, which downregulates PKB activity (not depicted).

Another interesting membrane Ca^{2+}/lipid-mediated binding signal transduction enzyme is cytosolic phospholipase A_2 ($cPLA_2$). Ca^{2+} and phosphorylation by MAP kinase are the most prominent regulators of $cPLA_2$ activity. Phosphorylation, which is found to be on Serine 505 (Lin et al., 1993), has to precede the Ca^{2+}-induced translocation for maximal EGF-induced activation of $cPLA_2$ (Schalkwijk et al., 1996). This phosphorylation step is probably necessary to release monomeric $cPLA_2$ from the inactive $cPLA_2$ clusters in the cytoplasma (Bunt et al., 2000). Submicromolar Ca^{2+}-concentrations induce a translocation and binding of $cPLA_2$ to membranes thereby allowing access to its substrates. The binding and activation is mediated by the N-terminal Ca^{2+}-dependent phospholipid binding domain (CalB or C2-domain) (Wijkander & Sundler, 1992; Nalefski et al., 1994; Perisic et al., 1998). This domain shares homology with the C2 domains first identified in the conventional isoforms of PKC and is found in a range of proteins like PLCγ and synaptotagmin (Clark et al., 1995). The C2 domain of $cPLA_2$ was shown to preferentially bind vesicles consisting of PC in the presence of physiological calcium concentrations. Binding of the C2 domain occurred irrespective of the saturation of acyl chains or the presence of carbonyl oxygens at *sn*-1 and -2 linkages. It has therefore been suggested that the $cPLA_2$ C2 domain primarily interacts with the headgroup of PC (Nalefski et al., 1998). The crystal structure of the CalB domain, (Perisic et al., 1998) of cPLA reveals an eight β-strand (type III) topology exposing two Ca^{2+} ion-binding sites that are surrounded by three connecting loops (CBRs). In a model for the membrane interaction of the CalB domain, CBR1 provides the initial binding by interaction with the hydrophobic portions of the lipid headgroup. CBR3 strengthens this binding by insertion into the membrane. A path of basic residues is thought to interact (weakly) with the negatively charged headgroups and, together with CBR1 and CBR3, constitutes the membrane-binding motif. One important feature of the membrane binding by the $cPLA_2$-C2 domain, as compared to C2 domains in other proteins, is that hydrophobic interactions prevail over electrostatic interactions (Davletov,

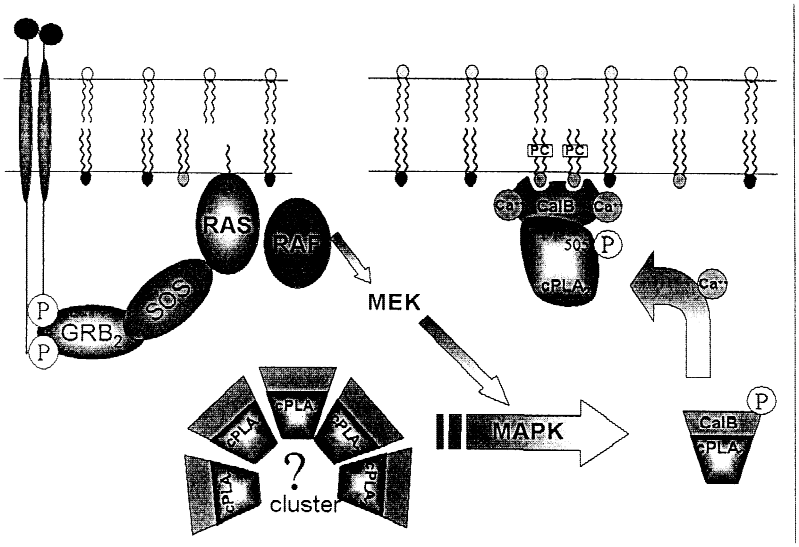

Fig. 3. $CPLA_2$ is present in the cytoplasm predominantly in clusters of which the identity is currently unknown. Upon activation of MAPkinase via the RAS-Raf-Mek pathway. $CPLA_2$ is phosphorylated at Ser505, which is proposed to release $cPLA_2$ as monomers, which will be translocated to the membranes upon binding of Ca^{2+}. It binds to the headgroup of PC, upon which preferential hydrolysis starts of arachidonic acid containing phospholipids.

Perisic & Williams, 1998). Notably, the $cPLA_{2\gamma}$ isoform lacks a C2 domain. Instead, membrane binding by this isoform is mediated by prenylation of its C-terminus (Underwood et al., 1998). In addition, the presence of a putative PH domain was proposed (Mosior et al., 1998). The high affinity and specific binding of $cPLA_2$ to PI (4,5) P_2 in vitro and the accompanying increase in activity suggests the presence of such a domain. Amino acids 271-283 revealed similarity with a portion of the PH domain of PLCγ and this region has been assumed to be the responsible motif. However, the functional implications of this putative PH domain and PI (4,5) P_2 binding remain to be established. In case of $cPLA_2$ translocation no role for the actin filament system could be found (Bunt et al., 1997). This is in line with the fact that $cPLA_2$ has no actin binding domain. It is of interest to note that the enzyme has a preference for arachidonoyl phospholipids, which are enriched in the cytoplasmic leaflet. This selectivity for arachidonoyl phospholipids is caused either by the loose packing in the membrane due to a high degree of unsaturation, or by selective recognition.

In conclusion the activation of $cPLA_2$ is present as inactive molecular complexes in clusters or aggregates of which the composition is not yet known. Upon activation of MAP kinase $cPLA_2$ is released from the clusters as monomers to only a small extent and translocate to membranes where the rise in Ca^{2+} is sufficient to induce the binding of two Ca^{2+} ions to the Calb domain. Then $cPLA_2$ bind to phosphatidylcholine and possibly PI (4,5) P_2, both lipids present in the inner leaflet of the membrane (Fig. 3).

In summary, lipids play an important role in signal transduction either by binding and/or activation of signal transduction molecules and as substrates of lipases, lipid kinases and phosphatases. Either they are constitutively present or they are locally induced in the cytoplasmic leaflet of the membrane upon receptor activation. We have exemplified this by the regulation of PLC, PKB and $cPLA_2$. Clearly, many other proteins and enzymes are translocated and activated to the membrane by lipid molecules, see for instance the reviews of Isenberg and Niggli (1998) and Liscovitch and Cantly (1994). It can be stated that heterogeneity in time and space will be one of the most intriguing parameters in membrane molecule functioning.

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