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COMMENTARY

PLATELET-ACTIVATING FACTOR RECEPTOR AND SIGNAL TRANSDUCTION

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Arachidonic cascade and platelet-activating factor

Platelet-activating factor (PAF,† 1-*O*-alkyl-2acetyl-*sn*-glycero-3-phosphorylcholine) [1–5] is a potent lipid autacoid which exerts a wide range of biological activities. This factor is involved in various pathologies such as bronchial asthma, endotoxin shock and other disorders [for review, see Refs. 6–10]. PAF is produced by two different pathways, but the major one is the so-called "remodeling system" where PAF is produced from the PAF precursor phospholipid (1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine) by the action of phospholipase A_2. Arachidonic acid and lyso-PAF are produced simultaneously, and arachidonic acid is further converted to various types of prostaglandins and leukotrienes (collectively termed eicosanoids) [11, 12], while lyso-PAF is converted to PAF by Ca^{2+}/calmodulin-dependent acetyltransferase. Therefore, it is reasonable to assume an interaction between PAF and eicosanoids: both act synergistically in some species of cells, and in others PAF exerts a variety of biological functions, through eicosanoid production (Fig. 1).

Evidence of a cell surface receptor for PAF

Since the discovery and structural identification of PAF, its specific receptor has been given much attention. Various observations concerning this receptor include the following:

(A) Strict structural requirement and stereospecificity for the bioactivity of PAF. An alkyl-ether bond at the C1 position of the glycerol moiety is necessary for activity, while replacement with an ester bond leads to a loss of the activity. Among various carbon-chain lengths, C16 was found to possess the highest activity. The acetyl moiety shows the strongest biological activity at the C2 position; the longer the acyl chain is, the less the activity. Phosphocholine, but not phosphoethanolamine, at the C3 position of the glycerol moiety is crucial for the related biological activity. The naturally-occurring *R*-chirality at C2 is active, while the stereoisomer (*S*-form) is inactive. Such a structure–activity relation revealed the presence of a specific receptor [13–15].

(B) Specific antagonists. Three classes of known PAF antagonists include phospholipid analogs, natural products, and the chemically-synthetic compounds as deduced from *in vitro* screening efforts. The structure and potencies of various antagonists are described in Refs. 13–15.

(C) Specific and saturable bindings of radiolabeled PAF and WEB 2086. More direct evidence was obtained from binding experiments, using either radiolabeled PAF or antagonists. The binding sites for [^{3}H]PAF were found in human [16–19] and rabbit [20] platelets, human leukocytes [21], human lung [22], and rat liver membranes [23]. The tritium-labeled PAF antagonist [^{3}H]WEB 2086 seems to be the best available compound for the binding experiments because it is metabolically stable, and the nonspecific binding is much lower than PAF [24]. An antagonist with a much higher specific radioactivity would be needed to identify high-affinity binding sites.

(D) Driving second messenger systems. Following activation of the receptor, several events occur which include phosphoinositide breakdown (PI turnover [25–32]), an activation of phospholipase A_2 [33–36] and D [37–39], inhibition of the adenylate cyclase system [33], and activation of protein kinase C [40, 41] and tyrosine kinase [42–44]. All these results strongly suggest that PAF binds to specific surface receptors in the cell membrane, but several lines of evidence suggest the presence of an intracellular receptor [45, ‡].

Cellular events following receptor activation

There is a growing body of evidence that PAF stimulates the breakdown of polyphosphoinositides. Thus, generated products, inositol trisphosphate (IP_3) and diacylglycerol (DG) serve as second messengers, playing roles in intracellular Ca^{2+} mobilization and activation of protein kinase C, respectively. Although some reports showed that

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† Abbreviations: PAF, platelet-activating factor; PI, phosphatidylinositol; TPI, triphosphoinositide; DPI, diphosphoinositide; IP_3, inositol trisphosphate; DG, diacylglycerol; IAP, islet-activating protein; and cRNA, complementary RNA.

‡ Hwang S-B and Wang S, Wheat germ agglutinin-potentiated specific binding of platelet activating factor (PAF) to human platelet membranes: Possible existence of endogenous modulator of PAF receptor and the intracellular PAF receptor. In: *Taipei Satellite Symposium on Platelet Activating Factor*, Taipei, May 4–7, 1989.

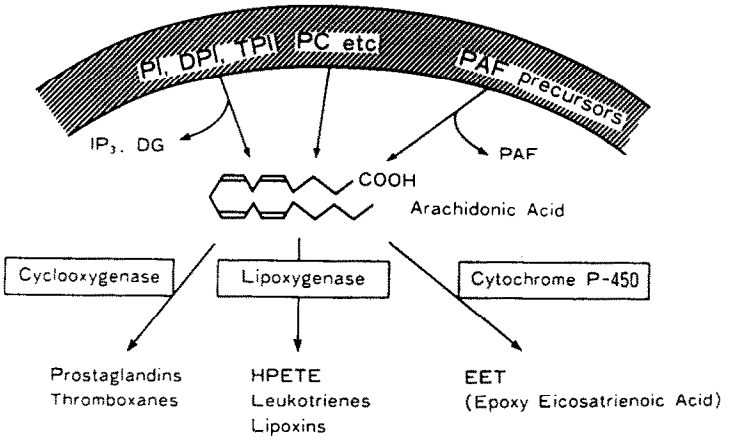

Fig. 1. Arachidonic acid cascade and PAF formation. Abbreviations: PI, phosphatidylinositol; DPI, diphosphoinositide; TPI, triphosphoinositide; PC, phosphatidylcholine; HPETE, hydroperoxy-eicosatetraenoic acid; and EET, epoxyeicosatrienoic acid.

Ca^{2+} channel opening is essential for PAF action [46], most PAF responses can be attributed to the mobilization of Ca^{2+} from internal Ca^{2+} stores by IP_3 formation. Ca^{2+}-influx can be a secondary consequence of intracellular mobilization of Ca^{2+} by IP_3, since the microinjection of IP_3 into *Xenopus laevis* oocytes [47] and mouse lacrimal acinar cells [48] leads to an influx of Ca^{2+} through a Ca^{2+} channel. Tyrosine phosphorylation is induced by PAF in rabbit platelets [42, 44] and in human polymorphonuclear leukocytes [43]. In the former study, it was concluded that PAF stimulates phosphorylation of $pp60^{c\text{-}src}$ tyrosine kinase and causes it to rapidly translocate from the cytosol to the membrane in rabbit platelets [44]. The expression of early responsive genes such as c-*fos*, TIS-1 and c-*jun* was noted using epidermoid carcinoma A-431 cells [49] and human neuroblastoma cells [50].

Characterization of the PAF receptor and signal transduction system

Despite the extensive work to solubilize and purify the PAF receptor from various cell sources [51–53], reports of purification of the receptor have not been published. The speculated M_r range is between 50 and 220 kDa [for review, see Ref. 14]. The difficulty in purification relates to the fact that the PAF receptor is labile and is a highly-integrated membrane protein. Pertinent antagonists for affinity purification have not been developed yet. A homology-screening approach by the PCR (polymerase chain reaction) technique was not feasible because there were no known sequences for receptors of related lipid autacoids.

Molecular cloning and expression of the PAF receptor. Using a *X. laevis* expression system, several types of receptor cDNAs have been cloned. The cloning strategy is summarized in Fig. 2. Briefly, oocytes efficiently translate exogenous mRNA, perform the correct posttranslational modifications, and transport those exogenous proteins to appropriate cellular compartments [54]. Thus, the functional membrane receptor appears on the cell surface 2–3 days following mRNA injection, when the microinjected mRNA contains the one which encodes the membrane receptor. The presence of the Ca^{2+}-dependent Cl^- channel in oocytes offers another advantage, since it permits the detection of the intracellular Ca^{2+} mobilization with high sensitivity by the voltage-clamp method (Fig. 2a). Thus, it is possible to isolate the gene for a receptor which is coupled with PI turnover and increases intracellular Ca^{2+}. By conventional methods, a cDNA library was constructed using a size-fractionated mRNA from guinea pig lung, a cDNA synthesis kit (Pharmacia) and a phage vector, λZap II (Stratagene) [55]. Making use of T7 RNA polymerase, cRNA were synthesized *in vitro* in the presence of the cap analog from 10 pools of 30,000 independent phage clones. Thus produced cRNA, instead of mRNA from tissues was microinjected, ligand-dependent Cl^--current was electrophysiologically screened, and positive fractions were divided by sibling (Fig. 2b). Starting from the 3×10^5 independent clones from the guinea pig lung cDNA library, a single clone with a 3-kbp insert was finally obtained which was subjected to DNA sequence analysis [55]. Human homolog of PAF receptor was isolated from the leukocyte library [56]. An 0.8 kb *Sma I* fragment of the guinea pig lung receptor cDNA was radiolabeled by a multi-primer labeling system (Amersham), and was used as a probe for hybridization. A single clone (1.8 kb) coding for the PAF receptor was isolated, and both receptor cDNAs were expressed in oocytes and COS-7 cells for the analysis of their pharmacological properties (see below) [55, 56].

Properties of cloned PAF receptors. The nucleotide and deduced amino acid sequences of PAF receptors from two species are given in Refs. 55 and 56. Both guinea pig lung and human leukocyte receptors are composed of 342 amino acids (M_r about 38 kDa, without calculating the sugar moiety). The overall identity of amino acids was 83%. More than 90%

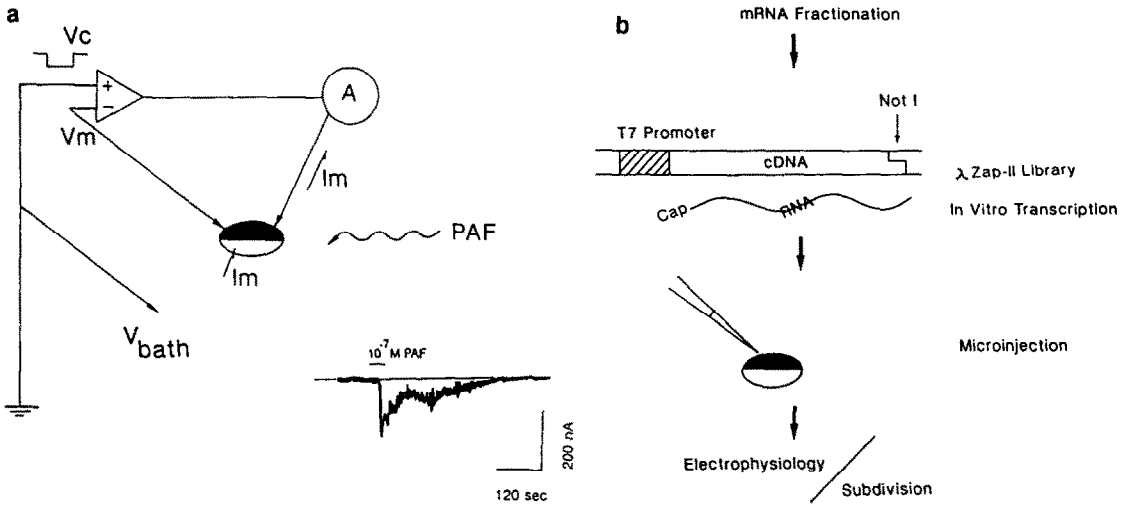

Fig. 2. Strategy of expression cloning of PAF receptor. (a) Intracellular Ca^{2+} elevation was monitored by Cl^- channel opening. The Cl^- current was measured by the voltage clamp method. Since the reversal potential of Cl^- was around −20 mV, the inward current was observed when the voltage was clamped at −60 to −100 mV. A typical trace is shown using 10^{-7} M PAF. (b) cDNA library synthesis, *in vitro* transcription and microinjection of cRNA into oocytes. Starting from 10 × 30,000 independent clones, a single clone coding for PAF receptor cDNA was obtained by sibling.

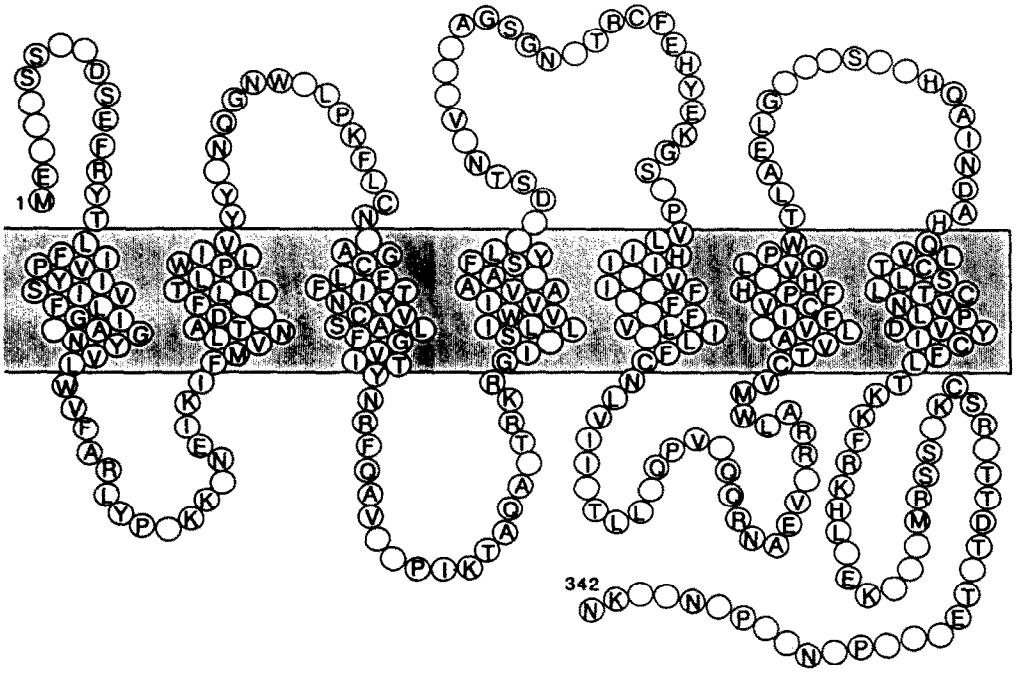

Fig. 3. Distribution of the conserved amino acids in the human leukocyte and guinea pig lung PAF receptors. A putative transmembrane structure is illustrated. The conserved amino acids between two receptors are illustrated in a single letter. The extracellular space and the cytoplasm are shown at the top and bottom, respectively [56].

homology was observed in the transmembrane spanning domains, while only 70% was conserved in loops connecting each segment. Conserved amino acids between two species are illustrated in Fig. 3, in a single letter form [55]. Hydropathy analysis revealed that these receptors have seven putative transmembrane segments, characteristic of the G-protein-coupled receptor superfamily. As shown in

Fig. 3, conserved are two cysteins in the second and the third extracellular loops, possibly making a disulfide bond, three prolines in the sixth and seventh transmembrane domains, eight residues of serine/threonine and one cystein in the C-terminal cytoplasmic loop. In addition, three threonine residues are present in the second and third cytoplasmic loops. Some of these serine/threonine residues can be a phosphate acceptor, relating to the homologous desensitization by certain kinds of kinases or a receptor-specific kinase. In contrast to the guinea pig lung receptor and other G-protein-coupled receptors, the human leukocyte receptor lacks the N-glycosylation site at its N-terminal extracellular loop (Fig. 3). A completely identical amino acid sequence as human leukocyte PAF receptor [56] has been reported by Ye *et al.* [57] who has cloned PAF receptor cDNA from HL-60 cells.

To elucidate the pharmacologic properties of the cloned PAF receptors, the clones were expressed in *X. laevis* oocytes or mammalian cells (CHO cells and COS-7 cells). As shown in Fig. 4a, exposure of oocytes to PAF elicited concentration-dependent increases in the Cl^- channel opening. The EC_{50} values were around 10 nM, under our assay conditions. 1-*O*-Octadecyl-2-acetoamide-2-deoxy-glycero-3-phosphocholine, a weak agonist, is less potent by two orders of magnitude [55]. The Ca^{2+} increase of PAF was inhibited by equimolar concentrations of PAF antagonists, CV-6209 and Y-24180 (Fig. 4b). Binding assays were carried out with [^{3}H]WEB 2086 using COS-7 cells transfected with guinea pig lung cDNA (Fig. 4c) and the human leukocyte PAF receptor cDNA. Scatchard analysis shows a single entity of the binding sites for these receptors. Binding parameters, B_{max} and K_d values, are 6.9 pmol/mg of protein and 6.4 nM for guinea pig lung receptor, and 9.2 pmol/mg of protein and 8.1 nM for human leukocyte receptor, when transiently expressed in COS-7 cells. These K_d values agree well with those found using various cells and tissues. Receptor activation is susceptible to homologous desensitization with PAF application, as determined by oocyte Cl^- current [55, 56]. Since the response produced by IP_3 remained unchanged before and after PAF application, the decrease in the response may be due to either down-regulation of the receptor or to the impairment of receptor/G-protein/effector coupling, but not to the depletion of the Ca^{2+} store [56].

Distribution of PAF receptor mRNA. In guinea pig tissues, PAF receptor mRNA is most abundant in leukocytes, followed by lung, spleen and kidney [55]. Although hardly detectable in Northern blots, other organs such as brain have a functional PAF receptor [58]. In the guinea pig kidney, the PAF receptor mRNA and B_{max} values obtained using [^{3}H]WEB 2086 were most abundant in the cortex, followed by the outer and inner medulla [59]. In human cells, PAF receptor is predominant in peripheral granulocytes. When EoL-1 cells (eosinophilic leukemia cells) are stimulated with interleukin-5 and granulocyte macrophage colony stimulating factor, they express a fairly large amount of PAF receptor mRNA, together with morphological changes and increased staining of eosinophil peroxidase [56]. A similar differentiation was observed by treatment with *n*-butyrate (Izumi T and Shimizu T, unpublished data). Muller *et al.* [60] reported that the PAF receptor mRNA expression is induced when HL-60 cells are differentiated to a macrophage phenotype by 1α,25-dihydroxy-vitamin D_3. In contrast, HEL cells, either non-stimulated or stimulated with phorbol 12-tetradecanoyl 13-acetate, show a faint band [56].

Signal transduction through G-protein(s) and PI turnover. Involvement of G-protein(s), following receptor activation, was directly demonstrated by microinjection of the inactive GTP analog, GDPβS into oocytes. Over 70% of PAF-elicited Cl^- currents was inhibited by the injection, while injection of water had no effect [56]. In transfected COS-7 cells and oocytes microinjected with receptor cRNA, PAF at 10^{-8} M rapidly increases the formation of IP_3 [56]. All these results confirm that ligand-activation of PAF receptor stimulates PI turnover through G-protein(s). The type of G-protein(s) involved in the PAF responses may differ from cell to cell. It was reported that the PAF responses in rabbit platelets [30] and the human monocytic cell line U937 [32] are resistant to the islet-activating protein (IAP) treatment, while responses in rabbit neutrophils [30], human macrophages [61] and human platelets are sensitive to IAP treatment. Thus, both IAP-insensitive (possibly G_q), and -sensitive (G_i or G_o) may be involved in the signaling pathway of PAF. Nakajima *et al.* [36] obtained evidence that PAF causes a K^+ channel (K_{ACh}) opening in guinea pig atrium through arachidonate metabolites. This channel opening by PAF is inhibited by IAP treatment of atrial cells [36]. Furthermore, the activation of the K^+ channel is blocked by 2-bromopheacylblue (BPB), a phospholipase A_2 inhibitor or AA-861, a 5-lipoxygenase inhibitor. This finding supports an earlier report by us that LTC_4 is a possible intracellular messenger to activate a K^+ channel ($I_{K\cdot ACh}$) in the guinea pig atrium [62].

Perspectives—What we now know and what is still unknown

We have described herein our recent work on molecular cloning of the PAF receptor from guinea pig lung [55] and human leukocytes [56]. We now know that these receptors belong to the superfamily of G-protein-coupled receptors. They are among the smallest receptors known, both having only 342 amino acids. The receptors couple with G-protein(s) and cause PI hydrolysis, as determined in cRNA-injected *Xenopus* oocytes and mammalian cells (COS-7 cells and CHO cells) transfected with receptor cDNAs. The following questions are being asked in the ongoing studies.

(1) Are there subtypes for the PAF receptor? By various pharmacologic experiments, mostly based on the order of potency of various antagonists [14], leukocytes and platelets have been shown to have different subtypes of receptor. However, by extensive homology screening of several libraries from different cell sources under low stringency, we have yet to find any evidence of subtypes of the receptor. Since

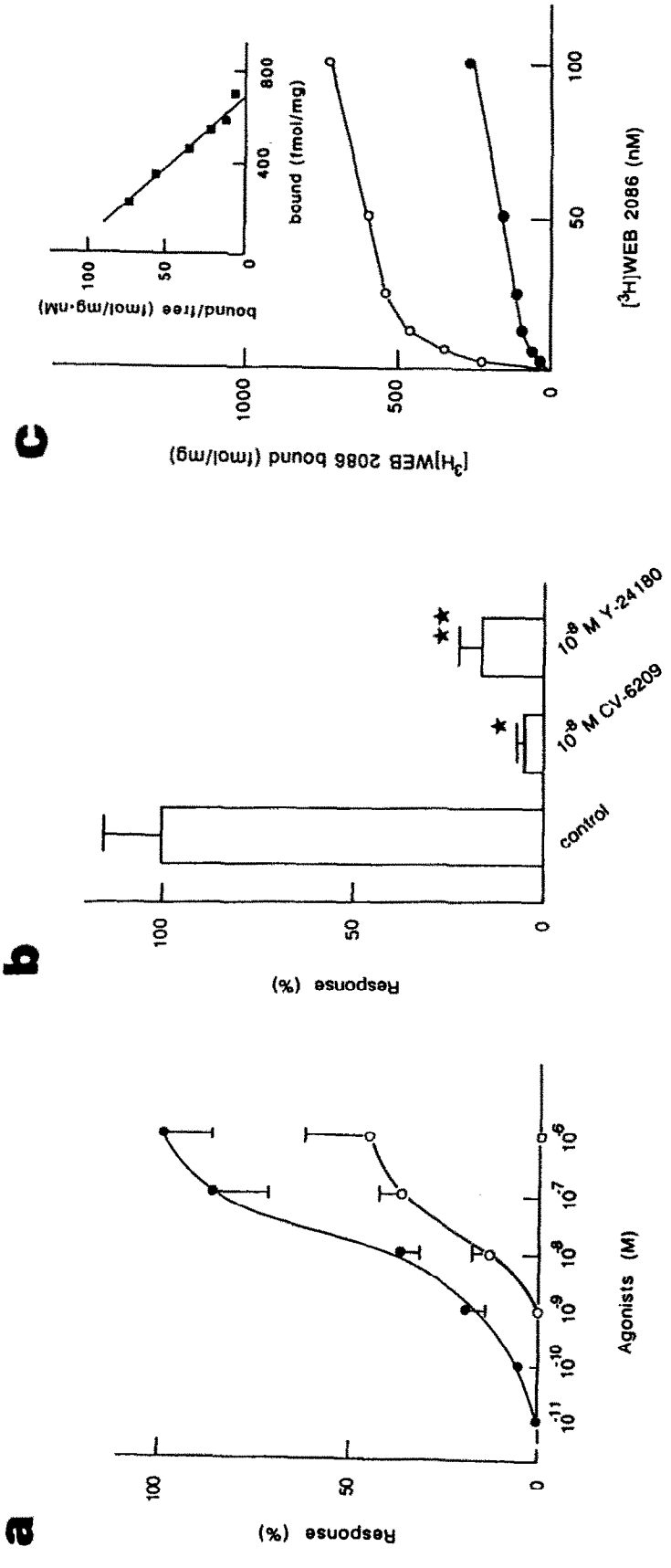

Fig. 4. Pharmacologic properties of cloned guinea pig PAF receptor. (a) Concentration-dependent curve of PAF (●) and 1-*O*-octadecyl-2-acetamido-2-deoxy-glycero-3-phosphocholine (○) in eliciting electrophysiological responses on oocytes. Values are means ± SEM. (b) Inhibition of PAF-elicited inward current by antagonists CV-6209 and Y-24180. Vertical columns and bars are means ± SEM, respectively. Key: * and **, $P < 0.01$ (Student's *t*-test). (c) Binding characteristics with [^{3}H]WEB 2086 of oocytes expressing PAF receptor encoded by cloned guinea pig receptor cDNA. K_d and B_{max} values are 6.4 nM and 6.9 pmol/mg of protein, respectively. *Reprinted by permission from Nature* **349**: 342–346 [55].

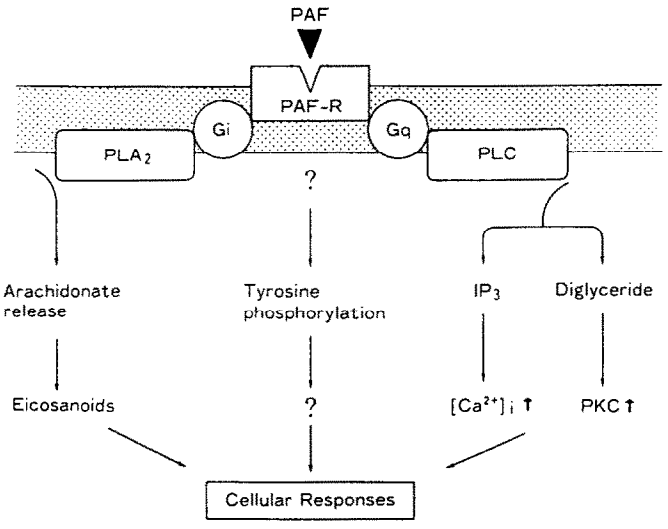

Fig. 5. Proposed model of PAF action for cellular responses. In addition to the scheme, the inhibition of the adenylate cyclase and the activation of phospholipase D can occur. PLA_2 and PLC denote phospholipase A_2 and PI-specific phospholipase C, respectively.

a single receptor can cause multiple conformational changes, depending on G-protein coupling or physicochemical alteration of the lipid bilayer membrane, these events may explain the different behavior to antagonists.

(2) Is there an intracellular PAF receptor? This argument mainly derives from the unusual behavior of PAF which is retained within the cell after synthesis, and readily internalizes in the membrane of the activated platelets [63] or neutrophils [64], when applied exogenously. The oocyte expression system as well as the patch clamp method are expected to provide the confirmative evidence as to whether or not intracellular receptor is present.

(3) How does tyrosine phosphorylation come out through the G-protein-coupled receptor? The cross-talk between PI turnover and tyrosine phosphorylation is observed with various growth factors [platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)] and other ligands (e.g. bradykinin, thrombin and formyl-methionyl-leucyl-phenylalanine). Receptors for these growth factors were isolated, and the mechanism of cross-talk has been clarified recently [65–71]. The receptors for PDGF and EGF are composed of a polypeptide chain(s) with essentially one transmembrane spanning domain. The extracellular domain has a ligand binding site, whereas the cytosolic domain contains both tyrosine kinase activity and the auto-phosphorylation site. Thus, by the application of the ligands, phosphorylated receptors can physically associate with a protein family possessing the SH2 (*src* homology) domain. This family includes various proteins (GAP, GTPase activating protein, *src*, *crk*) including phospholipase Cγ. Thus, "activated" phospholipase C initiates PI turnover. The problem, here, is how the G-protein-coupled receptor causes tyrosine phosphorylation (Fig. 5). Alternatively, there might be another yet unidentified PAF receptor which is linked to tyrosine phosphorylation.

(4) What type(s) of G-protein(s) and effectors couple to PAF receptor in each cell?

(5) What is the mechanism of PAF receptor desensitization? While there may be some phosphorylation of serine/threonine residues in the cytoplasmic loop, the kinases involved in phosphorylation of the receptors remain to be identified.

(6) Which peptide sequences or domains are responsible for recognition of each part of the PAF structure, and which part transduces the signal to G-proteins?, and finally

(7) How are second messengers transmitted to the nucleus to activate expression of early responsive oncogenes, and cellular responses such as proliferation and differentiation?

Described here is an update of the progress made, particularly focused on our recent work related to PAF receptor cloning and elucidation of the primary structure. The coverage is not intended to be exhaustive, and references have been pertinently selected. Readers should refer to excellent review articles on this topic [6–10, 13–15].

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