Introduction
Phospholipases catalyse the cleavage of membrane phospholipids and thereby generate second messengers that participate in intracellular signal transduction processes or act as precursors of tissue hormones. Cytosolic phospholipase A₂ (cPLA₂) is an 85 kDa enzyme that cleaves arachidonic acid at the sn-2 position of the phospholipid [1,2]. The intracellular location of the enzyme enables it to mediate receptor-regulated release of arachidonic acid [3]. Arachidonic acid is the precursor for prostaglandins, thromboxanes and leukotrienes, and lysophospholipid can be metabolized to yield platelet-activating factor. These substances exhibit diverse physiological, tissue-specific activities by participating in haemostatic and inflammatory responses and in the regulation of vascular tone, blood pressure, uterine and glandular function.

The activity of cPLA₂ is regulated by an increase in the intracellular Ca²⁺ concentration and by phosphorylation. Ca²⁺ mediates binding of the enzyme to phospholipid structures without being involved in the catalytic mechanism itself. An increase in the intracellular Ca²⁺ concentration triggers translocation of cPLA₂ to cellular membranes through a Ca²⁺-dependent lipid-binding motif [2,4]. cPLA₂ contains a consensus sequence for mitogen-activated protein kinase (MAPK), Pro-Leu-Ser-505-Pro. Ser-505 is phosphorylated by p42 MAPK in vitro and in cells co-expressing cPLA₂ and p42 MAPK, as determined by the comparison of wild-type cPLA₂ with mutated Ser-505→Ala-505 cPLA₂ [5]. Phosphorylation by p42 MAPK increases the intrinsic activity of the lipase by 2- to 3-fold as measured in vitro using phosphatidylcholine vesicles or sonicated liposomes as substrate [5,6]. Concomitant activation of p42/p44 MAPK and cPLA₂ is observed in many cells. cPLA₂ is also phosphorylated in vitro by protein kinase C and by protein kinase A. However, depending on the conditions of the lipase assay, phosphorylation by protein kinase C has little if any effect on cPLA₂ activity; phosphorylation by protein kinase A does not alter cPLA₂ activity [5,6].

Regulation of cPLA₂ and arachidonic acid release in human platelets
More than 10 years ago, Halenda et al. [7] had already observed that phospholipase A₂ activity in platelets leading to the release of arachidonic acid might be regulated by phosphorylation. The arachidonic acid release stimulated by Ca²⁺-ionophores is synergistically enhanced by phorbol esters. These are tumour promoters that can activate protein kinase C because of their structural similarity with diacylglycerol which is the physiological activator of most of the protein kinase C subtypes. The synergy between phorbol ester and Ca²⁺-ionophore in the release of arachidonic acid is reversed by the protein kinase inhibitor staurosporine [8] or the more specific inhibitor of protein kinase C, Ro 31-8220 [9], indicating that this enhancement of arachidonic acid release is due to the activation of protein kinase C rather than to possible toxic effects of phorbol esters. Moreover, the specific inhibitor of the p42/p44 MAPK activating pathway, PD 98059, also reverses this synergy [10]. Thus, the synergistic effect of phorbol ester on Ca²⁺-induced arachidonic acid release is mediated by p42/p44 MAPK that are activated downstream of protein kinase C. Participation of p42/p44 MAPK in this synergistic response is a characteristic of the regulation of arachidonic acid release; the phorbol ester-induced 5-hydroxytryptamine release from platelets, for example, is not blocked by PD 98059 [10].

The combination of a Ca²⁺-raising agent with a protein kinase C activator is often used to mimic cellular responses to physiological stimuli that activate Ca²⁺ and protein kinase C downstream of phospholipase C. Many physiological stimuli that bind to G protein-coupled or tyrosine kinase-coupled receptors on the platelet surface lead to activation of phospholipase C and to subsequent increases in the intracellular Ca²⁺ concentration and protein kinase C activation. However, the powerful platelet stimulus thrombin regulates arachidonic acid release through a mechanism that differs from the response elicited by Ca²⁺-ionophore and phorbol ester.
Thrombin cleaves a short peptide from the extracellular N-terminus of the thrombin receptor, thereby activating a $G_q$ protein which leads to stimulation of phospholipase Cβ. p42/p44$^{mapk}$ are activated in thrombin-stimulated platelets with maximal activation at 1 and 2 min [9]. This response is inhibited by preincubation with the protein kinase C inhibitor Ro 31-8220 or with the inhibitor of the Raf–MAPK kinase (MEK) interaction upstream of p42/p44$^{mapk}$, PD 98059, demonstrating that p42/p44$^{mapk}$ activation is dependent on protein kinase C and Raf–MEK [9, 10]. Both compounds are therefore useful tools with which to investigate the causal relationship between p42/p44$^{mapk}$ and cPLA2 activation. In contrast to the results obtained with Ca$^{2+}$-ionophore and phorbol ester, arachidonic acid release is not decreased in the presence of Ro 31-8220 or PD 98059 [9, 10]. In agreement with the arachidonic acid release data, $^{32}$P-phosphorylation of cPLA2 is also not significantly altered in the presence of PD 98059 or Ro 31-8220. These results demonstrate that other kinase(s) than protein kinase C and p42/p44$^{mapk}$ phosphorylate cPLA2 in thrombin-activated platelets.

Phosphorylation of cPLA2 and activation of arachidonic acid release by another physiological platelet stimulus, collagen, also occurs independently of protein kinase C and p42/p44$^{mapk}$ [9, 10]. The collagen receptor is structurally different from the thrombin receptor and it couples to a tyrosine kinase for early signalling events [11] but activation of the arachidonic acid cascade seems to be achieved by similar kinase pathways in thrombin- and collagen-stimulated platelets.

It is interesting to note that there are cellular differences in the importance of phosphorylation for the release of arachidonic acid. In Chinese hamster ovary cells that overexpress cPLA2, preincubation with the general kinase inhibitor staurosporine blocks release of arachidonic acid stimulated by ATP [12]. In contrast, staurosporine does not inhibit the release of arachidonic acid in thrombin-stimulated human platelets although it fully inhibits $^{32}$P-phosphorylation of cPLA2 [9]. In addition, staurosporine has no effect on the Ca$^{2+}$-ionophore-stimulated arachidonic acid release [8]; the Ca$^{2+}$-ionophore triggers the largest arachidonic acid release response in human platelets but only weakly phosphorylates cPLA2 [9]. Thus, phosphorylation seems not to be necessary for the activation of cPLA2 in human platelets.

Indeed, phosphatase-treated cPLA2 exhibits substantial lipase activity in vitro [12]. It might be possible that a large increase in Ca$^{2+}$, as seen in thrombin- or ionophore-stimulated platelets, overrides the small increase in intrinsic activity of cPLA2. Phosphorylation is nevertheless expected to be of physiological importance especially at the beginning of haemostatic events where weaker stimuli, such as ADP and collagen, cause primary activation of platelets. The release of thromboxane A2 from platelets amplifies the activation cascade and leads to strong and fast activation of platelets so that the injury to the blood vessel wall is quickly blocked by a blood clot.

**Regulation of cPLA2 by stress-activated protein kinases**

The search for the kinase that phosphorylates cPLA2 and regulates its activity in platelets was facilitated by the description of another selective kinase inhibitor, SB 203580 [13]. SB 203580 inhibits a member of the stress-activated protein kinase (SAPK) family, p38$^{mapk}$ (SAPK2a; [14]), and its recently described isoform p38β (SAPK2b; [15]). SAPKs share the substrate consensus sequence with MAPK and are therefore candidate kinases to phosphorylate Ser-505 of cPLA2. SAPKs are stimulated by a range of stress stimuli such as heat shock, UV light, chemical stress, and oxidative stress. They are also stimulated by physiological activators, for example by tumour necrosis factor-α and interleukin-1. Human platelets contain p38$^{mapk}$, and this kinase is activated by thrombin, collagen and the Ca$^{2+}$-ionophore A23187 but not by phorbol ester [16–18]. SB 203580 inhibits activation of platelet p38$^{mapk}$ in vivo with an IC$_{50}$ of approximately 0.2 μM [18], which is similar to values obtained from studies in vitro (IC$_{50}$ = 0.6 μM; [14]).

SB 203580 decreases $^{32}$P-phosphorylation of cPLA2 by approximately 50% in platelets stimulated with 1 unit/ml of thrombin for 2 min [17]. Analysis of the shift in the electrophoretic mobility of cPLA2 that is caused by the phosphorylated protein shows that full inhibition of phosphorylation at low thrombin concentrations is overcome at higher thrombin concentrations and by longer stimulation times [17]. Collagen-induced cPLA2 phosphorylation is nearly fully inhibited by SB 203580 as determined from $^{32}$P-labelled platelets and from electrophoretic
mobility shift experiments [18]. However, when platelets are stimulated with collagen in the absence of the cyclo-oxygenase blocker indomethacin, allowing the formation of thromboxane A2 and secondary stimulation, 32P-phosphorylation of cPLA2 is substantially increased and SB 203580 only partially reduces this phosphorylation [18]. Under the same conditions, PD 98059 has no effect on cPLA2 phosphorylation [18] demonstrating that p42/p44
 phosphorylate cPLA2 when platelets are stimulated by the combination of collagen and thromboxane A2.

Although the inhibition of cPLA2 phosphorylation by SB 203580 causes a decrease in the intrinsic activity of the lipase which can be measured in vitro [17,18], it is difficult to monitor an effect on arachidonic acid release from intact platelets. In the presence of 1 mM of extracellular Ca2+, SB 203580 and its more potent analogue SB 202190 do not decrease the arachidonic acid release in platelets under conditions where phosphorylation of cPLA2 is blocked [17]. In the absence of additional Ca2+ in the platelet buffer, a small decrease of arachidonic acid release is detectable from collagen-activated platelets [18]. Ca2+ is an important regulator of cPLA2 activation, and high Ca2+ levels in platelets that are activated by powerful stimuli could override the effect of phosphorylation.

A role for p38
 (and possibly subtypes) in the regulation of cPLA2 is not only observed in platelets. Phosphorylation and activation of cPLA2 in human neutrophils treated with tumour necrosis factor-α is regulated by p38
 rather than by p42/p44
 [19]. However, it is also possible that both p38
 and p42/p44
 contribute to cPLA2 phosphorylation. For example, stimulation of platelets by cross-linking of the low-affinity IgG receptor Fcγ-RII leads to cPLA2 phosphorylation that is partially inhibited by PD 98059 and by SB 203580 [18]. Interestingly, the Ca2+-ionophore A23187 stimulates p38
 (and not p42/p44
) but causes only a small phosphorylation of cPLA2 [9,17] indicating that p38
 activation does not always lead to cPLA2 phosphorylation.

The first map of human cPLA2 phosphorylation sites was obtained from the baculovirus/insect cell (Sf9 cells) expression system [20]. Human cPLA2 that was overexpressed in Sf9 cells was found to be phosphorylated in okadaic acid-treated cells. The phosphorylation sites were mapped after tryptic cleavage of cPLA2 that had been purified by immunoprecipitation and SDS/PAGE. Protein sequencing revealed that cPLA2 expressed in Sf9 cells was phosphorylated at four serine residues, namely Ser-437, Ser-454, Ser-505 and Ser-727. Of these four serines, Ser-505 and Ser-727 are highly conserved between different species [20,21]. The kinase that phosphorylates Ser-727 might be a basotrophic kinase such as protein kinase C or protein kinase A. Protein kinase C and protein kinase A phosphorylate cPLA2 in vitro [5,6]. Protein kinase C incorporates phosphate into at least five different sites [6] but whether Ser-727 is phosphorylated is not known.

Phosphopeptide mapping by two-dimensional TLC has the disadvantage that it is difficult to obtain quantitative data. Phosphopeptides may adhere to the tube wall during the repeated steps of sample concentration and removal of buffer salts after the tryptic digest. Therefore, Gelb and co-workers set up a microbore HPLC column that would allow cPLA2 samples containing only a few thousand c.p.m. (in contrast with 100000 c.p.m. obtained from cPLA2 over-expressed in Sf9 cells) to be analysed [22]. This new method is sensitive enough to detect phosphopeptides from samples containing less than 1000 c.p.m.

The first phosphopeptide map of endogenous cPLA2 by microbore HPLC was constructed using cPLA2 isolated from thrombin-stimulated human platelets [22]. Thrombin causes incorporation of 32P into two tryptic phosphopeptides which co-elute with standards bearing the Ser-505 and Ser-727 phosphorylation sites. 32P-labelled standards were generated by tryptic digests of cPLA2 phosphorylated in vitro by p42
 and of cPLA2 isolated from okadaic acid-stimulated Sf9 cells.

Thrombin stimulates phosphorylation of Ser-505 and Ser-727 to an equal extent. The partial decrease of thrombin-induced cPLA2 phosphorylation by SB 203580 [17] affects both phosphorylation sites [22]. Given the high degree of selectivity of SB 203580 towards p38
 and its isoform p38β [14,15], inhibition of both phosphorylation sites by SB 203580 suggests that phosphorylation at Ser-727 is either sterically dependent on Ser-505 phosphorylation, or that the kinase phosphorylating Ser-727 is activated downstream of p38
. Since p42
 phosphoryl-
ates cPLA₂ in vitro only at Ser-505 [5,22], it is unlikely that the SB 203580-sensitive SAPK directly phosphorylates Ser-727. SB 203580 only partially inhibits thrombin-induced cPLA₂ phosphorylation [17,22] although the inhibitor concentration used in these studies completely blocks activation of the in vitro substrate of p38mapk, MAPK-activated protein kinase 2 [22].

Recently, several new members of the SAPK family were described. SAPK3 and SAPK4, which are not inhibited by SB 203580 [23], are candidate kinases for cPLA₂.

Phosphorylation at Ser-727, the newly discovered phosphorylation site [20,22], probably occurs in conjunction with Ser-505 phosphorylation as there are several examples of both sites being phosphorylated in vitro. Incubation of platelets with collagen in a stirred solution and activation of HeLa cells with interferon-γ or arsenite triggers phosphorylation at Ser-505 and at Ser-727 [22]. In human monocytes that are stimulated by okadaic acid or zymosan, both sites are also phosphorylated [20]. In addition, exposure of mouse macrophages to phorbol ester or bacteria results in phosphorylation of several CNBr fragments of cPLA₂ including fragments bearing Ser-505 and Ser-727 [24].

**Conclusions**

The regulation of cPLA₂ in cells that are stimulated by physiological agonists is very complex. First, Ca²⁺ and serine phosphorylation contribute to the activation of cPLA₂. Secondly, several members of the MAPK/SAPK family are often activated by the same stimulus, but not all stimuli cause phosphorylation of Ser-505. Thirdly, there is a second phosphorylation site, Ser-727, and depending on the cell type possibly more sites, and neither the kinase(s) for these serine residues nor the functional impact of phosphorylation at these positions are known.

It is clear from the inhibitor work that the concomitant activation of certain MAPK/SAPK isoforms and the phosphorylation of cPLA₂ does not prove a causal link between the two events. If there are specific kinase inhibitors available, such as SB 203580 and PD 98059, it is possible to investigate the causal relationship of kinase activation and cPLA₂ phosphorylation. However, there are no specific inhibitors available for the newly described SAPK3 and SAPK4 or of c-Jun N-terminal kinase (SAPK1). This will make it particularly challenging to assess the roles of these enzymes.

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The structure of phospholipase C isoforms and the regulation of phosphoinositide hydrolysis

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The modular domains of phospholipases C

The second messengers Ins(1,4,5)P$_3$ and sn-1,2 diacylglycerol (DG) are produced by phospholipase C (PLC)-catalysed hydrolysis of the inositol phospholipid PtdIns(4,5)P$_2$ [1,2]. A significant body of experimental evidence now supports the contention that members of this extended family of enzymes are well-designed to perform this role in signal transduction. Isoenzymes of the three main families of PLC ($\beta$, $\gamma$, and $\delta$) all contain a series of common modules or domains which facilitate the reaction performed by PLC [3,4]. This modular structure contains an N-terminal pleckstrin homology (PH) domain, four EF hand repeats, a common active site containing two conserved regions termed X and Y boxes and a C-terminal C2 lipid-binding domain. Additional domains are found in the $\beta$ and $\gamma$ isoforms of PLC which are crucial to the particular mechanisms by which they are regulated (by heterotrimeric GTP-binding proteins and tyrosine kinases, respectively).

That this modular core of PLC is appropriate for the reactions catalysed by PLC is evident from the catalytic mechanisms by which these enzymes operate. Experimental evidence shows that all three families of PLC adhere to the surface dilution or dual-substrate hypotheses of PtdIns(4,5)P$_2$ hydrolysis [5–8]. In this kinetic description, which takes into account the fact that the reaction catalysed by PLC occurs in the two dimensions of a lipid–water interface formed at a membrane, the enzyme is considered to anchor itself to the lipid surface in a semi-stable manner using a part of the molecule distinct from the active site. Subsequently, a substrate molecule occupies the active site and is hydrolysed to products. A single residence time, during which the enzyme remains tethered at the membrane, may include hydrolysis of several substrate molecules as the enzyme moves over the surface of the lipid in a processive manner.

The role of the PH domain in anchoring PLC to membranes

The site of the enzyme which anchors it to the interface has not been conclusively identified, although for the $\delta$ isoform of PLC, there is good evidence that the PH domain fulfills this role. Thus, the PH domain has been shown to bind with significant affinity to phosphoinositides and their inositol phosphate head groups [9,10] and the crystal structure of this domain shows that this interaction is stabilized by several contacts formed between the first two variable loops of the PH domain and various points on the inositol phosphate [11]. Furthermore, the activity of a truncated enzyme which lacks a significant portion of the PH domain no longer shows any dependence on the mole fraction of PtdIns(4,5)P$_2$ in lipid vesicles and micelles [12], which suggests that this form of the enzyme does not become anchored at the lipid interface. In addition, micro-injection of various forms of PLC$\delta$1 into living cells followed by immunohistochemical analysis shows that PLC$\delta$1 becomes located at the plasma membrane of the cells in a PH domain-dependent fashion [13]. Taken together, these data imply that the PH domain is the membrane anchorage point for PLC$\delta$1 and that the enzyme obeys the dual-sub-

Abbreviations used: DG, sn-1,2 diacylglycerol; PLC, phospholipase C; PH, pleckstrin homology; PtdSer, phosphatidylserine.