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COMMENTARY

REGULATION OF RECEPTOR-MEDIATED ENDOCYTOSIS BY PHORBOL ESTERS

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I. INTRODUCTION

Phorbol esters are tumor-promoting agents whose primary intracellular receptor is the calcium/phospholipid-dependent protein kinase C, an intracellular serine/threonine kinase (reviewed in Ref. 1). Protein kinase C is normally activated by diacylglycerol produced during hormone-simulated activation of phospholipase C; activation involves the translocation of protein kinase C from an intracellular location to the plasma membrane. Phorbol esters, such as 4β-phorbol 12-myristate 13-acetate (PMA), can substitute for diacylglycerol, thereby activating protein kinase C in the absence of other agonists [2]. PMA-induced protein kinase C activation causes a variety of effects on cellular metabolism, differentiation, and growth (reviewed in Ref. 3). Furthermore, PMA-stimulated phosphorylation of growth factor receptors such as the insulin and epidermal growth factor (EGF) receptors modulates their ability to bind ligand and transmit metabolic and mitogenic signals [4–14].

PMA has been shown to affect the cellular distribution of a variety of cell surface receptors, and studies of PMA-stimulated receptor internalization have stimulated much discussion as to the role of serine/threonine phosphorylation in receptor-mediated endocytosis. This review will focus on the regulation of receptor trafficking by phorbol esters. An initial summary will describe the effects of PMA stimulation on the endocytosis and recycling of cell surface receptors. This will be followed by a discussion of possible mechanisms of PMA-stimulated alterations in receptor dynamics, and of several recent experiments using site-directed mutagenesis which directly test these mechanisms.

II. PMA EFFECTS ON RECEPTOR TRAFFICKING AND INTRACELLULAR DISTRIBUTION

A comparison of the effects of PMA on the internalization and recycling of various cell surface receptors is hampered by the fact that receptor internalization can be assessed using multiple parameters, such as ligand uptake, subcellular distribution, down-regulation as well as the actual rate at which receptors enter the cell. This can occasionally lead to apparently contradictory results: a decrease in ligand uptake may result from a markedly enhanced rate of receptor internalization, which in the absence of increased recycling could result in a decrease in the number of surface receptors available for ligand uptake. Conversely, an increase in the size of the intracellular receptor pool may result from a decrease in the recycling rate with no alteration in receptor internalization. Thus, care must be taken to identify the parameter which is actually measured.

Transferrin receptor

Among the best studied PMA-responsive receptor systems is that of the transferrin receptor, which mediates the uptake of iron bound to transferrin by internalizing through coated pits [15]. PMA was shown to induce the rapid ligand-independent down-regulation of the transferrin receptor; this down-regulation was subsequently shown to result from the translocation of surface transferrin receptors inside the cell [16–19]. In K562 cells, the PMA-induced change in receptor distribution was identical to that caused by transferrin itself [16]. Since PMA induced a hyperphosphorylation of the transferrin receptor, it was proposed that this phosphorylation might provide a signal for receptor internalization. A similar relationship between transferrin receptor phosphorylation and internalization was seen in PMA-treated HL60 leukemic cells: PMA caused an increase in transferrin receptor phosphorylation and a subsequent loss of surface transferrin binding [17, 19]. Both effects were potentiated by increases in intracellular calcium and were rapidly reversed upon withdrawal of PMA [17, 20]. Kinetic analysis showed that PMA increased the rate constant for receptor internalization by 2- to 3-fold, with a slight additional decrease in the rate constant for exocytosis [21]. These changes in the rate of endocytic parameters were sufficient to explain the observed redistribution of transferrin receptors.

A number of additional observations strengthened the correlation between PMA-stimulated phosphorylation of the transferrin receptor and its subcellular redistribution. Transferrin receptors at the cell surface were the primary substrate for PMA-stimulated phosphorylation, and their subsequent redistribution was blocked by the disruption of microtubule assemblies or by inhibitors of protein kinase C [21–23]. Trifluoperazine, an inhibitor of

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calmodulin and calcium/phospholipid-dependent kinases which paradoxically increases transferrin receptor phosphorylation, also stimulated transferrin receptor internalization [24]. Furthermore, infection of T cells with HTLV-1 blocked both PMA-stimulated transferrin receptor phosphorylation and redistribution [25].

Although PMA stimulates the internalization of transferrin receptor in erythroid and leukemic cell lines, leading to a net redistribution of receptors from plasma membrane to cell interior, its effects on receptor phosphorylation and distribution in other cell types are more varied and less well correlated. In A431 cells and cultured human lymphocytes, PMA increases transferrin receptor phosphorylation but does not affect its surface expression [26–28]. In contrast, PMA increases both the phosphorylation and the number of surface transferrin receptors in mouse 3T3 and Ltk^- cells, and Chinese hamster ovary (CHO) and Hep G2 cells [29–33], and increases cell surface transferrin receptor number in mouse tumor macrophages [34]. The effects of PMA on transferrin cycling may depend on cell differentiation, since phorbol-stimulated iron uptake in chick embryo myoblasts was abolished by transformation with Rous sarcoma virus [35]. Differentiation-dependent increases in transferrin receptor phosphorylation and cycling have also been reported in murine erythroleukemic cells [36, 37]. This complex pattern of effects makes a simple explanation of PMA-induced receptor redistribution difficult.

The similar redistribution of transferrin receptors caused by PMA or transferrin in K562 cells was consistent with the possibility that serine phosphorylation might play a general role in receptor-mediated endocytosis as a trigger for internalization [16]. Indeed, the rates of internalization and recycling of labeled transferrin and the uptake of ^{59}Fe were unchanged by PMA; this suggested that PMA and ligand may provide equivalent signals for receptor internalization, which occurred by the same mechanism with either stimuli. However, no increase in transferrin receptor phosphorylation was observed during ligand-stimulated internalization in HL60 cells or in sheep reticulocytes [17, 38]. Alternatively, since the transferrin receptor can internalize constitutively in the absence of ligand, it has been proposed that serine phosphorylation may regulate this process [15, 21, 39–41]. The identification of the PMA-stimulated serine phosphorylation site of transferrin receptor, serine 24, has facilitated the rigorous examination by site-directed mutagenesis of the role of serine phosphorylation of the transferrin receptor in the endocytosis of its ligand as well as its redistribution by phorbol esters [26]. These experiments will be described in section III.

EGF receptor

The binding of EGF to its receptor in cell membranes leads to the rapid autophosphorylation of the receptor on tyrosine residues, activation of a tyrosine kinase activity that is intrinsic to the receptor, as well as the internalization of the receptor–ligand complex into the cell (reviewed in Ref. 42). In most tissue culture systems, the internalized receptors are delivered to the lysosomes, where they are degraded along with their ligand [43, 44]. Recycling of the internalized EGF receptor, however, has been observed in hepatocytes [45]. Although EGF receptor internalization is accelerated markedly in the presence of ligand, the requirement for EGF-stimulated tyrosyl autophosphorylation during ligand-stimulated internalization is controversial [46–48].

The modulation of EGF receptor binding and autophosphorylation by PMA has been studied extensively. PMA treatment of intact cells has been reported to reduce EGF binding affinity in a number of cell types through the abolition of high-affinity binding sites [8–10]. PMA also inhibits the tyrosine autophosphorylation of the EGF receptor [7]. These effects are mediated presumably by the PMA-stimulated phosphorylation of the EGF receptor by protein kinase C at threonine 654, near the transmembrane domain of the EGF receptor [49–51]. However, a platelet-derived growth factor (PDGF)-stimulated increase in Thr-654 phosphorylation can occur in protein kinase C-down-regulated cells, suggesting that other serine/threonine kinases can utilize the EGF receptor as a substrate [52].

PMA-stimulated reductions in EGF-receptor number independent of changes in binding affinity have also been reported [53]. The effect of PMA on EGF-receptor internalization was directly examined in KB cells, where phorbol esters were shown to cause a rapid ligand-independent internalization of the receptor which led to a 50% decrease in surface receptor number [54]. Interestingly, this redistribution was transient, with receptor levels in the plasma membrane back to normal within 1 hr. Furthermore, the internalized receptors were not delivered to the lysosomes for degradation, as is the case during the ligand-stimulated internalization of EGF receptors in KB cells. Thus, PMA mimics ligand-stimulated internalization but not ligand-dependent intracellular routing of the EGF receptor.

Asialoglycoprotein receptor

The asialoglycoprotein (ASGP) receptor undergoes constitutive internalization via coated pits [55–57]. Although the receptor is phosphorylated on serine residues under basal conditions, with most of these receptors in an intracellular compartment, no increase in receptor phosphorylation occurs during ligand-stimulated internalization [58]. Serine phosphorylation of the receptor, therefore, does not appear to be required for ligand-stimulated internalization.

Modulation of the ASGP receptor by phorbol esters has been best studied in the Hep G2 hepatoma cell line, where PMA causes a transient decrease in receptor binding affinity, followed by a redistribution of 50% of the surface receptors inside the cell and a concomitant decrease in ligand uptake [33]. The redistribution is caused by a decrease in the recycling rate for internalized receptors, with little change in the rate of internalization [59], and is correlated with an increase in the serine phosphorylation of the receptor [58]. Pulse-chase experiments suggested that PMA-stimulated phosphorylation of ASGP receptors occurs primarily in the plasma membrane,

with subsequent movement of these receptors inside the cell. The slow turnover of these internalized phosphorylated receptors, as well as the observation that basally phosphorylated receptors are primarily intracellular, suggested that serine phosphorylation slows or prevents the recycling of internalized ASGP receptors. Thus, the behavior of ASGF receptors in Hep G2 cells, when contrasted with that of the transferrin receptor in HL60 cells, provides a striking example of how similar intracellular redistributions can be achieved through completely different PMA-stimulated mechanisms [21, 59].

Insulin receptor

The insulin receptor is a cell surface tyrosine kinase whose activity is stimulated by insulin binding (reviewed in Ref. 6). The mechanisms by which the activated receptor transmits metabolic and mitogenic signals are poorly understood. The insulin receptor undergoes insulin-stimulated as well as constitutive internalization (reviewed in Ref. 61); the requirement for tyrosyl phosphorylation of the receptor during ligand-stimulated internalization is controversial [62–66]. Like the EGF receptor, the insulin receptor is a substrate for PMA-stimulated serine phosphorylation by protein kinase C *in vivo* and *in vitro* [6, 11–13]. PMA-stimulated serine phosphorylation of the insulin receptor inhibits its insulin-stimulated tyrosine phosphorylation and tyrosyl kinase activity [11, 13, 14]. Inhibition of insulin binding to intact cells by PMA has also been reported, due to a decrease in insulin binding affinity [4, 5].

PMA stimulation of insulin uptake has been reported in a number of cell types [19, 67–69]. While this increase was attributed to a delay in insulin processing in Hep G2 cells, in vascular endothelial cells PMA caused an increase in the internalization rate of the insulin receptor [67, 69]. The net intracellular receptor pool did not increase, however, because of concomitant increases in the recycling rate of internalized receptors [70]. Interestingly, PMA did not stimulate ligand-independent internalization of the insulin receptor; this restriction of PMA-stimulated internalization to occupied receptors is so far unique to the insulin receptor [19, 69]. PMA, however, did increase the recycling rate of constitutively internalized insulin receptors in endothelial cells, suggesting that insulin was not required for PMA effects on receptor externalization [70].

Mannose 6-phosphate/IGF-II receptor

Independently studied for a number of years, the receptors for the mitogenic insulin-like growth factor II (IGF-II) and for lysosomal enzymes phosphorylated on mannose residues were shown recently to be identical [71, 72]. The IGF-II/mannose 6-phosphate (M-6-P) receptor is constitutively associated with clathrin-coated pits, and upon internalization has an intracellular itinerary which includes the endosome and the trans-Golgi (reviewed in Ref. 73). The receptor is phosphorylated on serine residues *in vivo* under basal conditions in a number of cell types [74–76]. Tyrosine phosphorylation has been observed in adipocyte plasma membranes but not in endothelial cells [76, 77].

Stimulation of vascular endothelial cells with PMA increased the serine phosphorylation of the IGF-II/M-6-P receptor, and led to a net translocation of receptors to the plasma membrane [76]. Interestingly, PMA also stimulated the internalization rate of the receptor, suggesting that the change in receptor distribution was due either to an even greater stimulation of the recycling rate, or to the recruitment of an intracellular receptor pool. The effects of PMA were abolished in cells in protein kinase C deficient cells, suggesting that this enzyme was responsible for the observed effects. This correlation between serine phosphorylation of the receptor and its intracellular distribution was opposite to those observed in insulin-stimulated adipocytes [74]. In the latter case, however, the sites of receptor phosphorylation were compatible with a casein kinase II-like serine kinase [78]. While it is not clear how the pattern of IGF-II/M-6-P receptor phosphorylation by these two serine kinases differs, it is possible that site-specific phosphorylation could allow additional levels of regulation of receptor trafficking. Alternatively, the different relationships between receptor phosphorylation and intracellular distribution could reflect cell-specific differences.

Immune system receptors and antigens

Recognition of antigen by T cells involves the T-cell receptor, a heterodimeric surface receptor which is complexed to the multimeric T3 antigen [79, 80]. Down-regulation of the T3/T-cell receptor complex is stimulated by the presentation of antigen by appropriate MHC-bearing cells or by anti-receptor antibody [81–84], and the complex also undergoes constitutive internalization [85]. Ligand stimulation also increase T3 δ-chain serine phosphorylation [79]. PMA treatment causes both an increase in T3/T-cell receptor phosphorylation on the γ- and ε-chains as well as a redistribution of receptors from the cell surface into the cell interior [79, 85, 86]. The redistribution is due, at least in part, to an increase in the receptor internalization rate, with the bulk of phosphorylated receptors inside the cell, suggesting that serine phosphorylation may stimulate T3/T-cell receptor internalization [85].

In contrast to PMA-stimulated internalization, increases in receptor internalization induced by anti-receptor antibody were not accompanied by changes in receptor phosphorylation [85]. However, internalization induced by divalent antibody binding to a receptor is not necessarily equivalent to that induced by receptor binding to a ligand [87]. Indeed, in other systems antibody-stimulated internalization has been observed even for mutant receptors which cannot undergo ligand-stimulated internalization [64].

The CD4 (T4) antigen is a T-cell membrane protein involved in interaction of $T4^+$ T cells with antigen-presenting cells bearing class II MHC antigens. Although the role of CD4 in T cell function is not clear, CD4 has been shown to bind MHC class II molecules [88]. However, the finding that the HIV gp120 envelope protein binds specifically to CD4 has attracted much attention to the receptor-like behavior of this T-cell surface antigen [89, 90]. CD4

has also been shown to be associated with the tyrosine kinase $p56^{lck}$, suggesting that it may play a role in signal transduction [91, 92].

A number of studies have shown that PMA modulates the expression of the CD4 surface antigen by T cells [93–97]. Acute stimulation of $T4^+$ T cells with PMA leads to the redistribution of surface CD4 molecules into the cell interior [98, 99]. This redistribution is coincident with a rapid and transient increase in the serine phosphorylation of CD4 [98, 100]. While this redistribution has been interpretated as an increase in CD4 internalization, the effects of PMA on the rates of CD4 internalization and recycling have not actually been measured; a change in either rate could cause the observed redistribution. A similar increase in CD4 phosphorylation and intracellular localization was observed during antigenic stimulation of $T4^+$ T cells, but not during nonspecific activation by phytohemmagglutinin [98, 100]. The PMA-stimulated effects may therefore reflect the physiological sequellae of CD4 interactions with its ligand. However, the binding HIV gp120, a known ligand for CD4, induces neither the phosphorylation nor internalization [100, 101]. Thus, the question of whether CD4 undergoes ligand-stimulated internalization remains obscure.

PMA affects the behavior of a number of phagocytic cell receptors. The C3b receptor, also called CR1, binds a proteolytic fragment of the complement component C3. Stimulation of polymorphonuclear leukocytes (PMNs), monocytes and macrophages with PMA rapidly increases the incorporation of [^{32}P]phosphate into CR1. Phosphorylation reaches maximal levels at 3 min and persists for up to 20 min [102]. PMA stimulation of phagocytic cells also causes a transient increase in surface CR1 number [103–105]. This increase persists at low PMA doses (4 ng/mL), but at doses of 16–30 ng/mL the number of surface CR1 molecules declines after 15–30 min [104, 105]. This decline represents the translocation of receptors inside the cell due to an increase in the ligand-independent internalization rate [103, 104]. The phagocytosis of C3b-coated erythrocytes by CR1 is also transiently stimulated by PMA [103, 105, 106]. Interestingly, calcium ionophores augment PMA stimulation of ligand-independent CR1 internalization but block PMA-stimulated phagocytosis, suggesting that these processes proceed by different mechanisms [107].

The surface expression of Fc receptors, which bind the Fc region of immunoglobulins, is affected in a biphasic manner by PMA; a transient increase in surface receptor number is followed by a decrease to below basal levels [105, 108]. While an increase in the phagocytosis of immunoglobulin-coated erythrocytes by PMNs is seen at 5 ng/mL PMA, an inhibition of phagocytosis is observed at higher doses [105, 109]. PMA stimulation of PMNs increases the surface number of the C3bi receptor (CR3), which binds a degradative fragment of C3b [105]. Phagocytosis of C3bi-coated erythrocytes is stimulated by PMA in cultured monocytes and PMNs, although this stimulation is transient in the latter cell type [105, 106]. The increased ability of PMA-stimulated PMNs to engulf opsonized particles correlated with an increase in CR3 clustering in the plasma membrane, suggesting that multivalent binding to opsonized particles might facilitate phagocytosis; no increases in Fc receptor clustering were observed [110]. However, PMA did not stimulate the association of CR3 molecules with clathrin-coated pits, suggesting that the internalization of CR3 may not be analogous to that of receptors for ligands like low-density lipoproteins (LDL) or transferrin [110].

Finally, the rate and extent of the constitutive internalization of Class I MHC antigens are stimulated by PMA in a number of lymphoid and macrophage lines [111, 112]. Although PMA stimulated the serine phosphorylation of class I MHC antigens in both lymphoid and non-lymphoid cells, increased internalization was only observed in the former [112]. Thus, like the transferrin receptor, PMA stimulation of MHC class I antigen internalization would seem to be cell specific. However, in another report, PMA did not stimulate the down-regulation of class I MHC antigens in human T lymphoblasts [86]. The groups reporting PMA-stimulated internalization in fact measured the disappearance of surface-bound anti-MHC antibody, which might reflect a pattern of receptor trafficking different from that induced by ligand or PMA alone [87].

Other receptors

PMA stimulates changes in the distribution of endocytosis of a number of other receptors. PMA decreases the number of plasma membrane LDL receptors in U-937 monocytes and in cultured human fibroblasts, and decreases LDL uptake in the latter [113, 114]. This decrease could be secondary to either a change in internalization rate or to reduced surface binding. An intracellular accumulation of the muscarinic acetylcholine receptor is caused by either ligand or PMA with identical kinetics [115]. In both cases, the change in receptor distribution was preceded by an activation of protein kinase C; receptor phosphorylation, however, was not demonstrated. PMA caused a rapid ligand-independent redistribution of tumor necrosis factor α into an intracellular compartment [116]; the rate of ligand uptake and degradation was not altered. A small PMA-stimulated decrease in PDGF-receptor binding was observed in Swiss 3T3 cells, although ligand uptake and degradation were unchanged [117]. PMA induced the phosphorylation of the interleukin 2 (IL-2) receptor as well as the loss of high-affinity IL-2 binding sites, although the total number of surface receptors was unchanged [118, 119]. Interestingly, this modulation of interleukin 2 binding by PMA was independent of receptor phosphorylation [118].

PMA effects on membrane dynamics

In addition to its effects on the intracellular distribution and trafficking of specific receptors, PMA causes more general shapes in cell architecture and membrane processes. Dramatic changes in cell shape and membrane spreading have been observed, as have increases in intracellular vesicles [120–125]. PMA affects the organization of microfilaments and

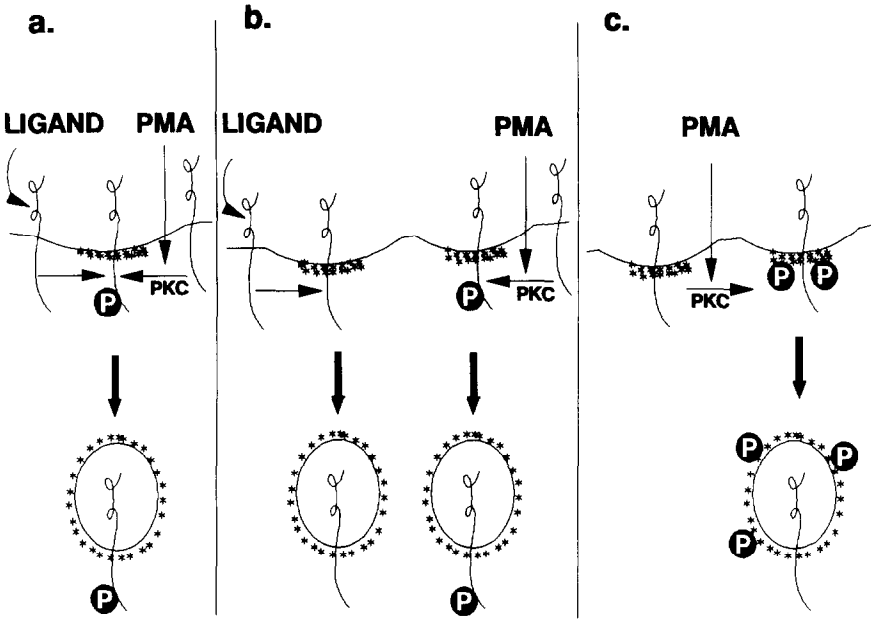

Fig. 1. Model of PMA-stimulated receptor internalization. The internalization of a prototypical cell surface receptor into coated vesicles is illustrated. Although the figure depicts the movement of receptors from uncoated regions of the membrane to coated pits (drawn as cross-hatched invaginations in the membrane), the proposed models apply equally well to receptors which constitutively reside in coated pits. (a) Ligand- and PMA-stimulated internalization proceed by the same mechanism, with serine phosphorylation of the receptor serving as a signal for internalization. PMA stimulates phosphorylation of the receptor by activating protein kinase C, while ligand stimulates receptor phosphorylation by an undefined mechanism. (b) Ligand- and PMA-stimulated internalization utilize different mechanisms. PMA stimulation of protein kinase C, leading to serine phosphorylation of the receptor, provides an alternative signal for internalization. (c) Ligand- and PMA-stimulated internalization utilize different mechanisms; in this case, PMA activates protein kinase C, which causes the phosphorylation of proteins in the endocytotic apparatus.

microtubules and their associated proteins, which may account for these changes [126, 127].

PMA stimulates pinocytotic uptake in neutrophils and macrophages, with an increase in the proportion of fluid-phase markers delivered to lysosomes and a decrease in recycling in the latter case [120, 128]. The changes in fluid-phase uptake were accompanied by increases in adsorptive endocytosis in neutrophils; the opposite was found in macrophages. PMA does not, however, stimulate membrane uptake in all cells: phagocytosis of rod outer segments by retinal pigmented epithelial cells is inhibited markedly by PMA [129].

III. MECHANISTIC INTERPRETATIONS OF PMA-STIMULATED CHANGES IN RECEPTOR TRAFFICKING

The great variety of effects caused by exposure of cells to PMA makes an overall understanding of protein kinase C-mediated changes in receptor trafficking difficult. However, these data have led to a number of hypotheses as to the role of serine/threonine phosphorylation in ligand-stimulated, constitutive and PMA-stimulated cycling. These hypotheses, which are illustrated in Fig. 1, can be divided into three models. The first assumes that PMA-stimulated trafficking is analogous to ligand-induced or constitutive internalization and recycling, with serine phosphorylation providing the trigger for internalization (Fig. 1a). In this model, PMA stimulation of protein kinase C mimics the signal for receptor internalization normally provided by ligand or, in the case of constitutive internalization, other cellular factors. One can then ask whether internalization, either ligand-stimulated or constitutive, requires receptor phosphorylation? The second and third models consider the possibility that PMA-stimulated trafficking may proceed by mechanisms which are alternative or additive to those used in ligand-stimulated internalization. Direct modulation of receptor phosphorylation might affect the rates of internalization and recycling without being required for these processes under basal conditions (Fig. 1b). In this case, one can again ask whether the PMA-stimulated changes in receptor trafficking require receptor phosphorylation. Alternatively, PMA stimulation of protein kinase C might alter the phosphorylation of any of the number of proteins involved in the function of coated pits and endosomes (Fig. 1c). In this model, PMA-stimulated internalization or recycling could proceed independently of receptor phosphorylation.

Requirement for serine/threonine phosphorylation in ligand-stimulated and constitutive internalization

While serine phosphorylation was initially proposed as a possible trigger for receptor internalization, studies in some systems have failed to detect ligand-induced changes in serine phosphorylation, which are kinetically compatible with internalization [16, 17]. In contrast, rapid ligand-stimulated increases in serine/threonine phosphorylation which correlate with ligand-induced internalization have been described for the β-adrenergic receptor and the T3/T-cell receptor complex [79, 81–84, 130]; conflicting results have been obtained on interleukin 2 (IL-2) stimulation of IL-2 receptor phosphorylation and internalization [131–133]. However, these studies cannot rule out a transient phosphorylation event preceding internalization. To directly examine the requirement for serine phosphorylation, several groups have evaluated the behavior of receptor mutants from which they have deleted PMA-stimulated serine/threonine phosphorylation sites.

In the receptors studied to date, deletion of PMA-stimulated phosphorylation sites does not appear to hinder constitutive or ligand-stimulated internalization. The transferrin receptor is phosphorylated in intact PMA-stimulated cells at serine 24 [26]. Four groups have independently constructed mutants in which serine-24 was substituted by alanine, glycine, or threonine. In all cases, while PMA-stimulated receptor phosphorylation was abolished, no change was seen in the uptake of transferrin or the kinetics of receptor cycling [29–32]. A similar study was conducted by mutation of the EGF receptor PMA-stimulated phosphorylation site, threonine 654 [49]. EGF stimulated the normal down-regulation of mutant receptor containing the substitution of alanine at residue threonine 654 [134]. Furthermore, a mutant in which four amino acids were inserted after residue 708 in the EGF-receptor kinase domain was also deficient in PMA-stimulated phosphorylation, whereas ligand-stimulated internalization was preserved [46]. While the requirement for serine/threonine phosphorylation of the insulin receptor for ligand-stimulated internalization has not been directly examined, recent studies have suggested that the primary PMA-stimulated phosphorylation site is threonine 1336 [135]; other prominent serine phosphorylation sites *in vivo* include serine residues 1293 and 1294 [136]. A truncation mutant lacking the C-terminal 43 residues, including threonine 1336, was shown to internalize normally in response to insulin [137]. The serine/threonine phosphorylation of the truncation mutant was not examined, raising the possibility of additional or alternative phosphorylation sites. Nonetheless, insulin receptor internalization clearly does not require phosphorylation at threonine 1336.

Requirement for receptor phosphorylation during PMA-stimulated internalization

While serine/threonine phosphorylation does not appear to be required for ligand-stimulated internalization, it might still be important for PMA-stimulated endocytosis. Alternatively, PMA might stimulate the phosphorylation of other components of the endocytic system, thereby regulating the internalization of cell surface molecules. PMA-stimulated internalization might also be a result of more widespread changes in membrane dynamics.

The requirement for serine phosphorylation in PMA-stimulated trafficking of the transferrin receptor was examined by site-directed mutagenesis of the protein kinase C phosphorylation site, serine 24 [26]. The reports of Klausner, May and their respective colleagues suggested that PMA-stimulated phosphorylation of the transferrin receptor could accelerate internalization [16, 17]. In contrast, studies of *in vitro* mutants of the receptor have shown that regulation of receptor movement by PMA could proceed even in the absence of phosphorylation at serine 24 [29–32]. However, none of the cell lines used in the mutation studies exhibit PMA-stimulated internalization of the wild-type receptor; in these cells PMA causes an increase in cell surface receptor number, as opposed to the decrease observed in K562 and HL60 leukemic cells. Thus, while the PMA-stimulated redistribution of transferrin receptors from intracellular to plasma membrane compartments in some cell lines may be independent of receptor phosphorylation, the requirement for phosphorylation in PMA-stimulated internalization has not been evaluated. The two processes may well involve different cellular mechanisms.

The requirement for receptor serine phosphorylation has been examined in a number of receptor systems which do undergo PMA-stimulated internalization. The endocytosis of *in vitro* EGF receptor mutants was studied in two different cell lines in which PMA stimulates the internalization without degradation of the wild-type EGF receptor. In contrast, PMA did not stimulate the internalization of a mutant EGF receptor lacking the threonine 654 phosphorylation site [49, 134]. Similar results were obtained for the CD4 antigen: wild-type molecules internalized after PMA stimulation, whereas mutant receptors in which a putative protein kinase C phosphorylation site was eliminated were unable to undergo PMA-stimulated internalization [101, 138]. Finally, PMA stimulation of the internalization of class I MHC antigens in lymphoid cells was lost in cells expressing a mutant antigen which was defective in PMA-stimulated phosphorylation [112]. However, this mutant MHC antigen also lacked a tyrosine residue which, by analogy to other receptor systems, may be important for coated-pit mediated internalization [139]. The behavior of this mutant in PMA-stimulated cells may therefore be complex. Nonetheless, in all cases where it has been examined, PMA stimulation of receptor internalization would seem to require receptor phosphorylation.

IV. CONCLUSION

The striking heterogeneity of the responses of cellular receptors to stimulation by PMA makes it difficult to formulate a broad hypothesis as to the action of this agent on receptor-mediated endocytosis. PMA stimulation activates the calcium/phospholipid-dependent protein kinase C, leading to the serine/threonine phosphorylation of various

intracellular substrates [3]. The varying results from different cell lines and receptors may reflect subtleties of kinase/substrate interaction, perhaps due to varying protein kinase C isozymes in different cell types [140].

Although serine phosphorylation does not seem to be required for ligand-stimulated internalization, there is evidence of the direct modulation of receptor trafficking by alterations in receptor phosphorylation. In some cases, such as the EGF receptor and the CD4 antigen, receptor phosphorylation may provide a signal which initiates internalization independently of other stimuli. In other cases, such as the insulin receptor, PMA-induced changes in receptor phosphorylation can be correlated with a modulation of the rates of ligand-stimulated internalization, but may be insufficient to independently drive receptor internalization. The asialoglycoprotein receptor provides a third alternative, in which PMA-stimulated receptor phosphorylation is correlated with a decrease in receptor recycling. The specificity of PMA-induced redistributions of different receptors in the same cell could be explained either by the variable ability of different receptors to be subsrates for activated protein kinase C, or by specific conformational changes in a given receptor which affect its association with other components of the endocytic machinery.

Site-directed mutations of the transferrin receptor, however, clearly illustrate that PMA-stimulated receptor redistributions can proceed independently of receptor phosphorylation. Interestingly, these phosphorylation-independent effects were observed in cells where PMA induces an increase in receptor exocytosis, rather than internalization. The variety of phorbol ester mediated effects on membrane turnover and cellular architecture raises the possibility that some of these changes in receptor distribution result from an overall mobilization of membrane components. Such widespread cellular events would be expected to affect numerous different receptors in the same cell, perhaps in a manner analogous to the insulin-stimulated exocytosis of multiple receptors (α-2 macroglobulin, transferrin, IGF-II) [74, 141]. If these redistributions were the result of a net flux of intracellular vesicles toward the plama membrane, then they should preferentially affect receptors which under basal conditions have sizable intracellular pools. Similarly, PMA-induced changes in the phosphorylation and function of components of the cellular endocytic machinery would be expected to alter the trafficking of multiple receptors. Indeed, variable phosphorylation of clathrin-associated adaptins has been reported to affect the efficacy of coated vesicle assembly (reviewed in Ref. 142). It is not clear whether PMA stimulation influences the phosphorylation of coated vesicle-associated proteins.

The regulation of receptor trafficking by phorbol esters remains a complex area of study. Although the stimulation of receptor internalization by PMA-induced serine phosphorylation does not appear to be a valid model of ligand-stimulated endocytosis, phorbol esters have been useful in shedding light on more subtle regulations of receptor trafficking. Site-directed mutagenesis of PMA-stimulated receptor phosphorylation sites is a promising approach toward illuminating the role of receptor serine/threonine phosphorylation in PMA-stimulated receptor redistributions. The mechanism by which PMA causes the redistribution of receptors independently of their phosphorylation state will require the determination of which proteins in the endocytic machinery are modulated by activation of protein kinase C.

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