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Mechanism of the Phorbol Ester-mediated Redistribution of Asialoglycoprotein Receptor: Selective Effects on Receptor Recycling Pathways in Hep G2 Cells

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SUMMARY

We have investigated the effect of phorbol dibutyrate on intracellular routing of the asialoglycoprotein receptor (ASGP-R) in a human hepatoma cell line, Hep G2. We have previously shown that this agent causes a net redistribution of 50% of cell surface receptors to the cell interior (Fallon, R.J., and A.L. Schwartz, *J. Biol. Chem.* **261**:15081–15089 (1986)). To explore the mechanism of this effect, we measured the rate constants of receptor and ligand movement during internalization, ligand-receptor uncoupling, sorting of ligand to degradative sites or return to the extracellular medium, and return of receptor to the plasma membrane. The rate of internalization of bound asialoorosomucoid (ASOR) is identical in phorbol ester-treated and control cells, over a range of ASOR concentrations from 5 to 125 nM. The pathway of ligand recycling returns approximately 30% of internalized ASOR undegraded to the extracellular medium; phorbol esters do not modify the extent of this pathway in Hep G2 cells nor the kinetics of recovery of undegraded ASOR in the medium ($t_{½}$ = 20 min). The rate of ligand-receptor uncoupling is similarly unaltered by phorbol esters, as measured by the amount of free ASOR that accumulates intracellularly and exits the cell after saponin permeabilization. In contrast, phorbol esters cause a rapid (<5 min) 50% decrease in receptor return to the cell surface from internal sites. This suggests that 1) phorbol esters interfere with selected specific sites in receptor and ligand pathways of receptor-mediated endocytosis and 2) the apparent net "internalization" of ASGP-R by phorbol esters results from an inhibition of receptor recycling to the cell surface and not from a direct stimulation of the internalization process.

The regulation of expression of cell surface receptors involves the integration of the pathways of biosynthesis, degradation, and receptor and ligand trafficking (1, 2). Studies of the asialoglycoprotein receptor (ASGP-R) have illustrated many routing pathways for both receptor and ligand in hepatocytes and the hepatoma cell line Hep G2 (3, 4). After internalization of the ligand-receptor complex from coated pits, clathrin-coated vesicles are formed for transport to internal endocytic vesicular structures. From there ligand may return to the plasma membrane in an undegraded form both free and bound to receptor (ligand recycling pathway). This pathway accounts for more than 20% of internalized ligand in hepatoma cells and is not disrupted by agents that neutralize intracellular acidic compartments (5). Most internalized ligand-receptor complexes, however, are sorted in an acidic tubulovesicular structure known as the compartment of uncoupling receptor and ligand (CURL) from which ligand is routed to lysosomal degradation. The uncoupled receptor, in contrast, returns to the plasma membrane via incompletely understood mechanisms that involve concentration within membrane vesicles, trafficking to the plasma membrane, and subsequent membrane-membrane fusion. Recent studies in hepatoma cells, fibroblasts, and Madin-Darby canine kidney cells have demonstrated an important role in protein sorting for a membrane-bounded structure in the region of the *trans* Golgi, called *trans* Golgi reticulum or *trans* Golgi network (6, 7). The involvement of this ultrastructurally defined compartment in the sorting and recycling of membrane receptors is incompletely understood.

It is apparent that recycling receptor systems provide numerous targets for modulation of receptor number at the cell surface. Several of these targets have been identified using selected pharmacologic agents. For example, agents that neutralize acidic compartments, such as the weak bases ammonium chloride or chloroquine or the ionophore monensin, decrease the number of surface receptors for many receptors that participate in receptor-mediated endocytosis (8–10) and document

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ABBREVIATIONS: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; CURL, compartment of uncoupling of receptor and ligand; DMSO, dimethyl sulfoxide; MEM, minimum essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate buffered saline.

the importance of acidic compartments in the processes of ligand uncoupling, receptor recycling, and ligand degradation. Similarly, an energy requirement late in the ASGP-R recycling pathway has been demonstrated (11).

Phorbol esters have been shown to modulate receptor number at the plasma membrane and cause a redistribution of the asialoglycoprotein receptor (12). It is unclear, however, where in the pathways outlined above phorbol esters are active or whether there is a unique site of action. Receptor hyperphosphorylation has been hypothesized to function as a signal directing receptor movement into the cell, because redistribution of phosphorylated receptor molecules from the plasma membrane to intracellular sites has been observed in multiple systems including the β-adrenergic receptor (13), transferrin receptor (14), and the epidermal growth factor receptor (15). Whether other nonreceptor targets of protein kinase C activation are critical to the observed alterations in receptor sorting is uncertain, however.

To approach these questions, we have examined the effect of phorbol esters on receptor/ligand pathways in the Hep G2 cell line, in which pathway kinetics are experimentally approachable and well described (16, 17). Our results show that phorbol esters selectively inhibit the pathway of receptor return to the plasma membrane and suggest that this effect, rather than accelerated receptor internalization, accounts for the final redistribution of ASGP-R to the cell interior.

Experimental Procedures

Materials

Human orosomucoid (provided by the American Red Cross) was desialylated and iodinated with ^{125}I as previously described (18). Dibutyryl 4-β-phorbol, 4-α-phorbol, 4-β-phorbol, primaquine, and saponin were obtained from Sigma. The phorbol esters were dissolved in dimethyl sulfoxide at a concentration of 200 μg/ml and stored at −20°C. The human hepatoma cell line Hep G2, clone a16, was used for all studies reported here. Maintenance of these cells has been described (16).

Methods

Surface receptor binding assay. The binding of ^{125}I-ASOR to Hep G2 cells at 4°C has been reported (19). Specific binding of iodinated ligand was defined as the difference between total and nonspecific binding. Nonspecific binding was determined by performing the binding assay in the presence of 200-fold excess unlabeled ligand. Nonspecific binding of ^{125}I-ASOR could also be calculated by means of an acid-stripping procedure. In this technique, radioactive ligand bound to the ASGP-R is selectively removed after the binding assay by incubation for 8 min at 4°C in PBS containing no calcium and 10 mM EDTA at pH 5.0. Either procedure results in 10–20% nonspecific binding for ^{125}I-ASOR.

Ligand internalization assay. Internalization of surface-bound radioligand has been described (16). Briefly, Hep G2 cell surface ASGP receptors were saturated with ^{125}I-ASOR at 4°C for 2 h, washed free of unbound ligand, and then temperature-shifted to 37°C for various times before return to 4°C. Ligand that remains bound to receptor at the cell surface is then determined by incubation with acid-stripping medium for 8 min at 4°C. ^{125}I-ASOR not removed by this washing procedure (internalized ligand) is determined after cell solubilization with 1N NaOH.

Ligand recycling assay. The exocytosis of internalized ASOR in an undegraded form via the pathway of ligand recycling (5) was studied after internalization of a single cohort of ligand molecules. After saturation binding of ^{125}I-ASOR, monolayers of Hep G2 cells were washed free of unbound ligand in PBS at 4°C. The cells were then allowed to internalize ligand for 4 min by incubation in prewarmed binding medium (MEM, 20 mM HEPES (pH 7.4), 0.1 mg/ml cytochrome *c*) at 37°C. After this period of ligand internalization, the cells were immediately chilled by immersion in PBS at 4°C. Ligand remaining on the cell surface was removed by exposure to PBS with no added calcium but containing 10 mM EDTA, pH 5.0 for 8 min at 4°C. The cells were then incubated in 1 ml exocytosis medium (binding medium containing 100 mM *N*-acetylgalactosamine (isosmotic replacement of NaCl)) at 37°C with or without phorbol dibutyrate in DMSO. The concentration of DMSO in control and phorbol ester-treated cultures never exceeded 0.1%. After incubation at 37°C, exocytosis medium was collected at the appropriate times, and radioactivity was determined in chilled 20% trichloroacetic acid/4% phosphotungstic acid insoluble and soluble (i.e., degradation products) fractions. Cells were solubilized in 1 ml of 1 N NaOH and subsequently assayed for total protein and radioactivity in a Packard gamma counter.

Assay for ligand-receptor uncoupling. Confluent Hep G2 cells were washed and incubated with 50 nM ^{125}I-ASOR at 37°C in binding medium. At the appropriate time, replicate dishes of cells were immersed in PBS at 4°C to inhibit further ligand internalization. The ^{125}I-ASOR bound to surface ASGP receptor was removed by treatment of the cells for 10 min at 4°C in acid-stripping medium as described above. The cells, which contained only iodinated ligand within the cell, were then treated with 0.1% saponin in binding medium for 30 min at 4°C. This detergent permeabilizes but does not solubilize cell membranes, enabling macromolecules as large as 2×10^5 kDa to enter or exit the cell at 4°C (20). Saponin treatment does not cause the loss of membrane-bound molecules such as ASGP-R from Hep G2 cells (5). In addition, control experiments have shown that saponin in this concentration does not lead to loss of previously receptor-bound ligand from the cell or change the affinity of ligand for receptor (data not shown). The saponin medium was then collected after 30 min and the amount of unbound ^{125}I-ASOR determined. Data were expressed as percent of ligand uncoupled by dividing this result by the sum of unbound ^{125}I-ASOR and ^{125}I-ASOR remaining membrane-bound within the cell.

ASGP receptor return to plasma membrane. Primaquine 300 μM was added to Hep G2 cells grown in 35-mm dishes for 30 min at 37°C. Under these conditions, the ASGP receptor is rapidly lost from the plasma membrane and is sequestered in an intracellular pool (17). The kinetics of reversal of this effect were evaluated by measurement of ASOR binding activity at the cell surface after removal of primaquine from the binding medium. After primaquine treatment, the cells were washed twice in fresh binding media and incubated at 37°C for the indicated times. The cell culture dishes were then rapidly chilled to 4°C by immersion in PBS. Cells were stripped of ligand bound to cell surface receptors by a 10-min incubation in PBS containing no calcium and 10 mM EDTA pH 5. ^{125}I-ASOR at 50 nM was added to the cells for 2 h at 4°C to estimate cell surface receptor number. Unbound radioligand

was then removed, the cells washed in PBS four times, and the remaining radioactivity solubilized with 1 N NaOH.

Results

Effect of phorbol esters on asialoglycoprotein uptake. The human hepatoma cell line Hep G2 has approximately 2 × 10^5 receptors for asialoglycoproteins on the cell surface (16, 19). One-hour treatment with 200 nM phorbol dibutyrate reduces this by 50%, although total receptor number in the cell is unchanged (12). Table 1 shows that phorbol ester addition also results in 40%–50% decrease in ligand uptake by cells. The rate of total ligand uptake is linear in phorbol ester-treated cells and remains linear for at least 6 hr (data not shown). Within 2 hr under these conditions ligand uptake by the asialoglycoprotein receptor exceeds that which could be accounted for by a single round of endocytosis of the entire cellular complement of ASGP-R molecules (12). These findings suggest that receptor recycling continues in the presence of phorbol dibutyrate. The altered receptor distribution and decreased rate of total ligand uptake (Table 1), however, are evidence that ASGP-R and ligand transport pathways in Hep G2 cells may be inhibited by phorbol esters. To further examine this question, we have investigated the effect of phorbol dibutyrate on the recycling pathways for receptor and ligand during endocytosis of ASOR.

Internalization of receptor and ligand after exposure to phorbol esters. We performed experiments to test whether the observed ASGP receptor redistribution is due to a direct stimulation of receptor movement into the cell (internalization) or to inhibition of receptor recycling back to the cell surface from intracellular sorting compartments. Earlier experiments (12) had suggested there was no change in the internalization rate after phorbol ester addition. These studies, however, were performed at high, saturating concentrations of ligand, conditions that might produce maximal stimulation of receptor internalization. Figure 1 shows ligand internalization by Hep G2 at 37°C over a wide range of ligand concentrations in the presence or absence of phorbol dibutyrate. The replicate cell cultures were prebound before warming for 2 h at 4°C with the indicated ASOR concentrations, ranging from 5 to 125 nM. Under these conditions, saturation of ASGP receptor binding occurs at 50 nM. Figure 1 shows that progressively less ASOR is bound to receptor with lower, subsaturating concentrations of ligand in the external medium, that the rate of ligand internalization is similar under those circumstances where detectable ASOR is bound to the cell, and that phorbol esters at this concentration do not accelerate ligand internalization. The concentration of phorbol dibutyrate used (200 nM) is maximal with respect to its effect on ASGP receptor redistribution. In addition, other data (see Fig. 5 below) show that phorbol esters can demonstrate activity with regard to modulation of intracellular events within 1–2 min. Furthermore, pretreatment with phorbol esters at 37°C before ligand binding does not alter the results in Fig. 1 (data not shown), again suggesting the time of exposure to these agents was sufficient to produce a possible stimulatory effect.

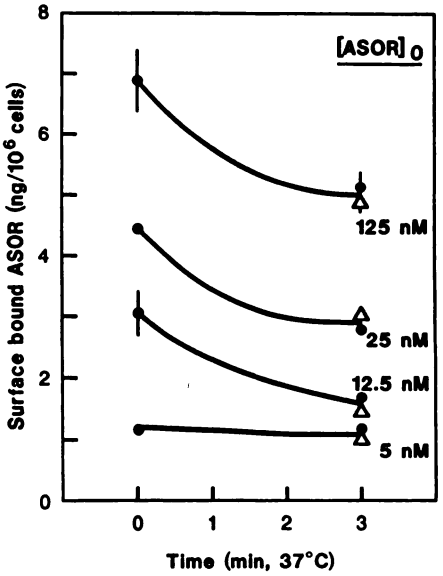

Fig. 1. Internalization of ^{125}I-ASOR by Hep G2 cells. Hep G2 cells were incubated for 2 h at 4°C with the indicated concentration of ^{125}I-ASOR. Cell monolayers were then washed to remove unbound ligand and warmed to 37°C in binding medium with 200 nM phorbol dibutyrate (*triangles*) or 0.1% Me_2SO (*circles*). At the indicated times dishes were quickly chilled to 4°C, rinsed with PBS, and incubated with 1 ml PBS containing no calcium and 10 mM EDTA, pH 5.0. The EDTA-releasable radioactivity represents receptor-bound ligand that has not yet been internalized from the cell surface. Results presented are means ± standard error for four replicate dishes.

We next determined the internalization rate of receptor during phorbol ester treatment. To focus on the pathway of ASGP receptor movement into the cell exclusive of the recycling pathways we exposed the cells to the weak base primaquine at a concentration sufficient to inhibit lysosomal and endosomal acidification and receptor return to the cell surface from intracellular sites (17). Previous studies in our laboratory demonstrated that primaquine had no inhibitory effect on ASGP-R internalization under these conditions. Figure 2 shows the rate of disappearance of ASGP-R from the Hep G2 plasma membrane during the first 10 min after primaquine addition at 37°C. The rate of this process in control cells (closed circles) is similar to the ligand internalization rate obtained in Fig. 1. Furthermore, there is no alteration of this rate when excess ligand (ASOR 500 nM) or phorbol esters are present. Thus receptor internalization occurs rapidly in Hep G2 cells at 37°C ($t_{1/2}$ = 3 min), even in the absence of exogenous ligand. This agrees with prior kinetic studies of this receptor in this cell line (16). In addition, Figs. 1 and 2 show that accelerated internalization of the receptor-ligand complex does not occur in Hep G2 cells after phorbol ester addition.

Pathway of ligand recycling in phorbol ester-treated

TABLE 1

Effect of phorbol dibutyrate on receptor-mediated uptake of ^{125}I-ASOR by Hep G2 cells

Hep G2 cells were incubated at 37°C with 50 nM ^{125}I-ASOR in binding medium containing 200 nM phorbol dibutyrate or 0.1% Me_2SO (control). At the indicated times, total ligand uptake was determined. This is defined as the sum of cell-associated radioactivity and acid-soluble radioactivity in the extracellular medium, assayed as described under "Experimental Procedures." Values from two independent experiments were combined and expressed as means ± standard deviation. PdB, phorbol dibutyrate.

	Total uptake		
	Control	Phorbol dibutyrate	PdB/control
	(ng ASOR/10^6 cells)		
Time of incubation			
30 min	16.7 ± 1.2	9.6 ± 1.6	0.57
60 min	22.1 ± 0.5	14.5	0.65
120 min	41.1 ± 5.0	24.5 ± 1.7	0.57

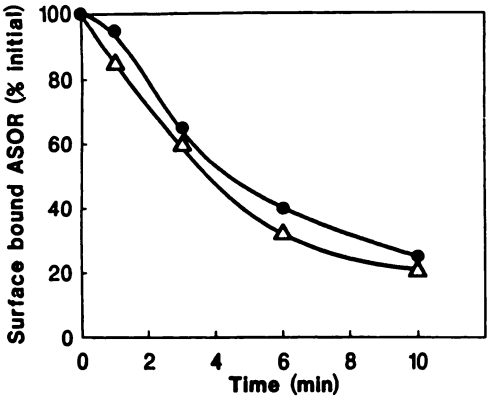

Fig. 2. Effect of phorbol esters on the rate of ASGP receptor internalization in primaquine-treated cells. At time zero, Hep G2 cells were treated with 300 μM primaquine in binding medium at 37°C for the indicated times. Also included in the medium was 0.1% Me_2SO (*closed circles*) or 200 nM phorbol dibutyrate (*triangles*). At each time duplicate dishes were immediately chilled (<20 sec) by immersion in PBS at 4°C, and binding assays with ^{125}I-ASOR were performed at 4°C for 2 h as described under "Experimental Procedures." Specific ligand binding to the cell surface at each time was calculated and plotted as percentage of binding at time zero in control cells. Specific binding to control Hep G2 cells under these conditions is 5 ng ASOR/10^6 cells.

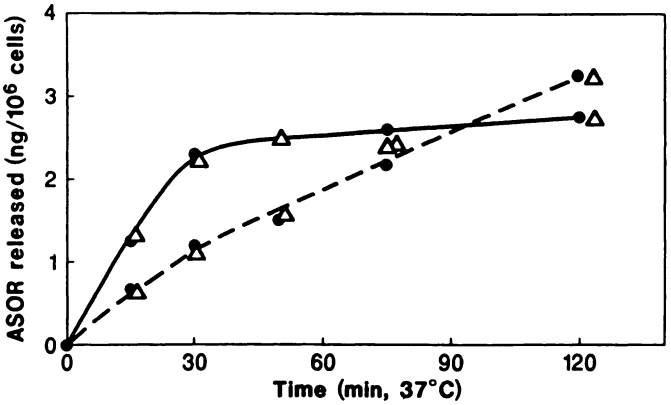

Fig. 3. ASOR recycling and degradation rates in Hep G2 cells exposed to phorbol esters. After saturation binding at 4°C in the presence of 200 nM ^{125}I-ASOR, monolayers were washed in PBS and allowed to internalize ligand at 37°C for 4 min. Cells were rapidly chilled to prevent further internalization, and ligand bound to the cell surface was removed by an 8-min incubation in 10 mM EDTA in PBS at pH 5, 4°C. Fresh medium containing 100 mM *N*-acetyl galactosamine was added, and exocytosis of undegraded ^{125}I-ASOR (*solid line*) as well as production of ligand degradation products (*dashed line*) were followed. Details of these methods are given under "Experimental Procedures." Duplicate dishes containing 200 nM phorbol dibutyrate (*triangles*) were analyzed at each time and compared with control (*closed circles*). In these experiments the total intracellular ^{125}I-ASOR at time zero was 7.5 ng/10^6 cells.

Hep G2 cells. We next examined the ability of phorbol dibutyrate to modulate the sorting and eventual fate of internalized receptor and ligand. Morphological studies demonstrate internalized ASGP receptor chiefly in two endocytic prelysosomal sorting compartments, CURL and *trans*-Golgi reticulum (3, 6). There is as yet no biochemical method for isolation and direct study of these compartments. Therefore, we employed the following indirect method for analyzing the rates of the sorting reactions. A single cohort of labeled ASOR molecules is allowed to enter the cell during a short (4 min) period of receptor-ligand internalization at 37°C. The cells are rapidly chilled to 4°C to inhibit further internalization, and iodinated ligand remaining at the cell surface is removed by incubation in PBS containing no calcium, 10 mM EDTA at pH 5.0. The internalized cohort of undegraded ^{125}I-ASOR at this point (time 0 min) is of equal size in both control and phorbol ester-treated cells. By incubating these cells at 37°C, with no external ^{125}I-ASOR, the fate of ligand can be followed independent of continued internalization into the sorting compartment. This experimental design eliminates a contribution resulting from phorbol esters' decrease in ASGP receptor number at the cell surface (12) which thereby secondarily reduces the amount of ligand internalized and degraded. This cohort is then followed for movement out of the sorting compartment(s), to either degradation within the lysosome or movement back to the plasma membrane with subsequent exocytosis of the undegraded ligand. This latter pathway, referred to as diacytosis or ligand recycling (21), has been well defined for Hep G2 cells in our laboratory using ASOR as ligand (5). Under these conditions, approximately 30% of internalized ASOR is returned to the extracellular medium in an undegraded form, accompanied through this pathway by ASGP receptor. Figure 3 shows the results of an experiment designed to ask whether phorbol ester alters the sorting of receptor-ligand complexes to this route and thereby modifies receptor distribution. The trichloroacetic acid-precipitable material appearing in the extracellular medium represents intact, undegraded ASOR (5). Figure 3 shows (solid line) that the exocytosis rate of ASOR is not affected by 200 nM phorbol dibutyrate. In this experiment the 4-min ligand internalization period occurred in the presence of phorbol esters. This ensures that the agent has adequate time to reach its intracellular site(s) of action and potentially alter a sorting reaction occurring very early in the internalization process (<4 min). In parallel experiments where internalization occurred in the absence of phorbol esters, identical curves were obtained (data not shown). These results suggest, therefore, that phorbol esters do not inhibit the entry of ASOR into the sorting compartment, its sorting to the pathway of ligand recycling, or its rate of movement through this pathway to eventual exocytosis.

Figure 3 also shows the extracellular accumulation of degradation products of the ^{125}I-ASOR cohort, as measured by solubility in 20% trichloroacetic/4% phosphotungstic acid (dashed line). As seen with ASOR recycling (solid line), no change in the rate of this process follows phorbol ester addition. The kinetics of ASOR degradation are different, however, from those observed for ASOR recycling to the cell surface. The process is linear for the 2 hr shown in this experiment and delivers (in degraded form) 1.8 ng ASOR/hr/10^6 cells to the medium. Further experiments show that ASOR degradation reaches a plateau at 3 h, at 6 ng/10^6 cells (70% of total internalized ligand at t=0). These results suggest that early in the ligand internalization pathway a proportion of ^{125}I-ASOR (here 30%) is distributed, by physical sorting or by biochemical marking, to a pathway involved in transport of the undegraded protein back to the plasma membrane. This actual transport and exocytosis reaction occurs rapidly ($t_{1/2}$ = 20 min). Since this reaction is complete by 45 min, at which time the degradation of ASOR is only 25% completed, the data also suggest that ASOR molecules distributed at early times to the degradation pathway are committed to eventual proteolysis and cannot regain access to the pathway of ligand recycling. Figure 3 shows that phorbol esters have no effect on this sorting reaction or on the rate of ligand movement through these pathways. In the

next section the reactions involved in the movement of ligand to lysosome are further examined.

Rate of ligand-receptor uncoupling in phorbol ester-treated cells. Since the bulk of flow for both receptor and ligand requires entry into an acidic prelysosomal compartment (CURL), uncoupling of ligand from receptor, and subsequent trafficking to lysosome or plasma membrane, the rate of ASOR uncoupling from ASGP-R intracellularly was determined after its uptake at 37°C. To assay this process phorbol ester-treated and control cells were allowed to bind and internalize ^{125}I-ASOR at 37°C. The cells were then chilled, washed, and permeabilized with 0.1% saponin to allow efflux of uncoupled ASOR molecules. Control experiments show that this detergent does not solubilize the ASGP receptor. Furthermore, ligand bound to receptor at the time of saponin addition is not stripped by this procedure if performed at pH > 7 in the presence of calcium (12 and data not shown). Figure 4 shows the results of this assay. As a positive control in this series of experiments 300 μM primaquine is added to duplicate cell cultures because this agent is known to neutralize acidic compartments and inhibit ligand-receptor uncoupling (17). Figure 4A shows the rate of intracellular accumulation of uncoupled ASOR with these treatments. The lower curve (open circles) has an equal concentration of ^{125}I-ASOR in the uptake medium, but 100 mM *N*-acetylgalactosamine has been included (isotonic replacement of NaCl). This agent inhibits binding of asialoglycoproteins to ASGP-R and thus provides an estimate of fluid phase ligand uptake. Primaquine (diamonds) inhibits the rate and extent of ASOR uncoupling by greater than 90% in control or phorbol ester-treated Hep G2 cells. Figure 4B incorporates the data from this uncoupling assay with those on total intracellular (membrane receptor-bound and uncoupled) ligand to demonstrate changes in percent uncoupled ligand with time after addition of phorbol ester or primaquine. Again, primaquine clearly inhibits the intracellular uncoupling of ligand and receptor as measured by the saponin permeabilization assay. In contrast, phorbol dibutyrate has no effect on this process; the percentage of internalized ligand that is uncoupled from the ASGP receptor is identical to control cells throughout the experiment. The plateau reached in panel B in phorbol ester and control cells at 45–60 min occurs despite continued uptake of ^{125}I-ASOR from the extracellular medium and likely represents the point at which degradation of uncoupled ASOR molecules equals the rate of delivery of newly internalized ligand into the cell. The data in Fig. 4 therefore support the conclusion that ligand-receptor dissociation, previously localized to CURL in the peripheral regions of the cell (3), is unaltered by phorbol esters. The action of phorbol esters on pathways of receptor mediated endocytosis distal to this uncoupling process is addressed below.

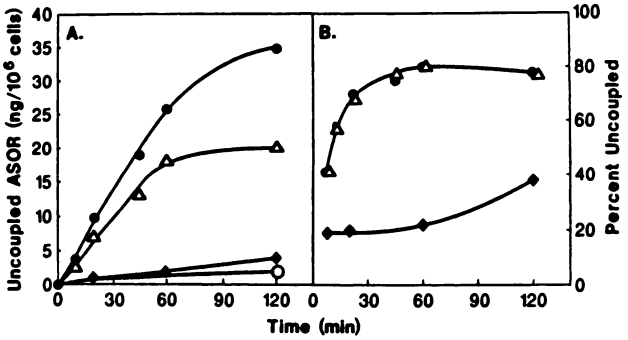

Fig. 4. Course of ligand-receptor uncoupling during continuous ASOR uptake at 37°C. Panel A, Hep G2 cells were incubated at time zero with fresh binding medium containing 50 nM ^{125}I-ASOR as well as 0.1% Me_2SO (*closed circles*), 200 nM phorbol dibutyrate (*triangles*), 300 μM primaquine (*diamonds*), and 100 mM *N*-acetyl galactosamine (*open circles*). At the indicated times, cells were washed in PBS at 4°C; surface receptor-bound ^{125}I-ASOR was removed by an acid-stripping procedure described under "Experimental Procedures," and intracellular uncoupled ASOR was determined by a 30-min incubation at 4°C with 0.1% saponin in binding medium. Panel B, percent of total internalized ligand uncoupled from ASGP receptor was calculated for each time by dividing the result in panel A by the total amount of ^{125}I-ASOR internalized at that time.

Effect of phorbol esters on the pathway of receptor return to the plasma membrane. After uncoupling of ligand, the ASGP-R concentrates within the tubular portions of the CURL sorting apparatus for eventual sorting into coated vesicles and return to the plasma membrane (6). There is no direct kinetic assay for this process. Consequently, we adopted the following strategy to examine this pathway: ASGP receptors were pharmacologically depleted from the plasma membrane by 30-min treatment with 300 μM primaquine, which causes a redistribution of 90% of cell surface receptor to intracellular site(s) (17). After removal of this agent, the kinetics of receptor recycling to the plasma membrane in the presence or absence of phorbol esters were examined. Figure 5A shows the rate of recovery of ^{125}I-ASOR surface binding sites in dimethyl sulfoxide- (closed circles) and phorbol dibutyrate-treated (triangles) cells after removal of primaquine. Control cells rapidly ($t_{1/2}$ 2–3 min) increase surface ligand binding to a level equalling that in Hep G2 cells before primaquine addition. Phorbol esters cause an immediate alteration in the number of receptor molecules that can be detected at the plasma membrane within the first 2 min. The steady state level attained by 7 min (50% of control) is maintained for at least 120 min (data not shown). This reduction in ^{125}I-ASOR binding seen in cells incubated with phorbol ester at 5–10 min after release of primaquine represents a decrease in surface ASGP-R molecules because 1) binding of ^{125}I-ASOR at greater than saturating concentrations

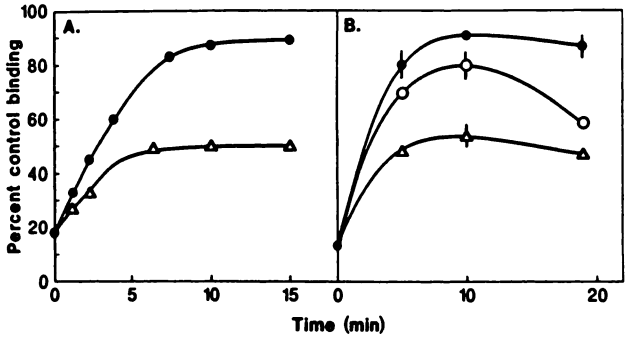

Fig. 5. Effect of phorbol esters on ASGP-receptor recycling to plasma membrane. Replicate 35-mm dishes of Hep G2 cells were pretreated with 300 μM primaquine in growth medium for 30 min at 37°C. At time zero in panels A and B, cells were washed twice in medium at 37°C, and fresh growth medium was added containing 0.1% Me_2SO (*closed circles*), 250 nM ASOR (*open circles*), or 200 nM phorbol dibutyrate (*triangles*). The washing procedure required 1 min for completion. At the indicated times dishes were immersed in PBS at 4°C and washed in PBS containing no calcium and 10 mM EDTA at pH 5.0. Surface ASGP receptors were then determined by performing a binding assay at 4°C with 50 nM ^{125}I-ASOR as described under "Experimental Procedures." Results are expressed as percent of specific ligand binding in control cells without primaquine pretreatment. Panels *A* and *B* show results from two independent experiments.

to Hep G2 also is decreased and 2) ligand binding affinity of the ASGP-R (6×10^{-8} M K_d) is unaffected at this time (data not shown). To further characterize the mechanism of the phorbol ester effect observed in Fig. 5A, 250 nM ASOR was added to control cells during the period of release from primaquine. At this concentration, external ASOR has been shown to rapidly reduce ASGP-R at the cell surface by binding to plasma membrane receptor causing receptor-ligand internalization (22). Figure 5B shows that 250 nM ASOR (open circles) reduces surface ASGP-R but with kinetics different from the phorbol ester-treated cells. At 5 and 10 min, surface ^{125}I-ASOR binding (open circles) is not substantially decreased from control levels. A 20% to 30% decrease in cell surface ligand binding is observed, however, at later times. This suggests that phorbol esters have a mechanism different from ligand for decreasing surface receptor in this experimental system. Since experiments presented above showed no stimulation of internalization rates with phorbol esters (Figs. 1 and 2), the data in Fig. 5 indicate that phorbol esters have an inhibitory effect on early events subsequent to primaquine removal, those occurring before receptor reinsertion at the plasma membrane.

The action of phorbol esters in this system was further addressed in Table 2, in which phorbol derivatives were tested for their ability to inhibit the appearance of ligand binding sites at the cell surface during the first 6 min after removal of 300 μM primaquine. Inhibition of ASGP receptor return to the plasma membrane in these studies was not observed with 4-α-phorbol or 4-β-phorbol, derivatives that do not promote protein kinase C activation. Table 2 further shows that the observed inhibition by phorbol dibutyrate is concentration-dependent, with no effect seen at concentrations below those required in other systems for activation of protein kinase C (23). This suggests that the major inhibitory action of phorbol dibutyrate on ASGP receptor return to the plasma membrane is mediated by protein kinase C. The ultimate phosphorylated protein target (receptor, enzyme or other kinase) responsible for this effect remains the subject of further study.

We conclude from the above data that the major location of phorbol ester inhibition of ligand and receptor movement in Hep G2 cells is on the pathway concerned with return of internalized receptors to the plasma membrane. The magnitude of this inhibition is sufficient to explain the observed redistribution of ASGP receptors by phorbol esters, as well as the continued ability (Table 1, Fig. 4) of these receptors to participate, although at a reduced level, in the recycling pathways of receptor-mediated endocytosis.

TABLE 2

Effect of inactive phorbol ester derivatives and phorbol dibutyrate on ASGP receptor recycling to plasma membrane after primaquine removal

Hep G2 cells were incubated with 300 μM primaquine for 30 min as described in Fig. 5, then washed and treated for 6 min with MEM containing the indicated agent and no primaquine. The increase in specific surface ^{125}I-ASOR binding during this period was measured as described under Experimental Procedures and compared with duplicate cells not incubated with primaquine (control initial (control$_i$)). The final DMSO concentration in all samples was 0.1%. Values shown are means ± standard deviation of triplicate or quadruplicate samples. One of two representative experiments was analyzed.

	New surface ^{125}I-ASOR binding sites 6 min	Control$_i$	*p* value
	ng/10^6 cells	%	
DMSO (0.1%)	8.9 ± 0.8	74	*
4-α-phorbol 400 nM	9.4 ± 0.6	78	0.35
4-β-phorbol 400 nM	8.4 ± 0.6	70	0.38
Phorbol dibutyrate			
0 nM	6.3 ± 0.5	70	*
20 nM	6.2 ± 0.1	68	0.80
80 nM	5.4 ± 0.2	59	0.06
200 nM	4.4 ± 0.3	49	0.01
800 nM	4.0 ± 0.1	45	0.003

* Two-tailed *t* test vs. control (DMSO alone in each experiment).

Discussion

In this study we have attempted to investigate the mechanism of phorbol ester-mediated redistribution of asialoglycoprotein receptor in a human hepatoma cell line. We examined the effect of phorbol esters on the kinetics of the individual pathways of receptor and ligand that have been described and that can be measured in intact cultured cells.

The results permit several major conclusions. Phorbol esters, at doses capable of maximally activating intracellular protein kinase C, inhibit the return of ASGP receptor molecules to the plasma membrane from an intracellular sorting compartment. This block is relatively specific in that there was no detectable effect of phorbol esters on other pathways of receptor mediated endocytosis. These findings are summarized in the diagram in Fig. 6. The inhibition of receptor recycling by phorbol esters is shown and compared with other known inhibitors of the receptor-mediated endocytosis pathways examined in this study and described above. This schematized drawing of a hepatoma cell also shows ligand in the ligand recycling pathway exiting the degradation pathway before entry into CURL. This represents one interpretation of available data on this pathway. Other possibilities that have not been eliminated, however, include the existence of a separate parallel ligand recycling pathway or actual entry of ligand-receptor complexes into CURL but with sorting to the receptor recycling pathway before uncoupling. In addition, the *trans*-Golgi reticulum has been omitted from Fig. 6. The possible relevance of this structure to ligand-receptor pathways has not been fully defined and is discussed in more detail below.

These findings and conclusions are consistent with prior observations on phorbol ester modulation of ASGP receptor physiology. A reduction in cell surface receptors to a level approximately 50% of that in control cells occurs during the

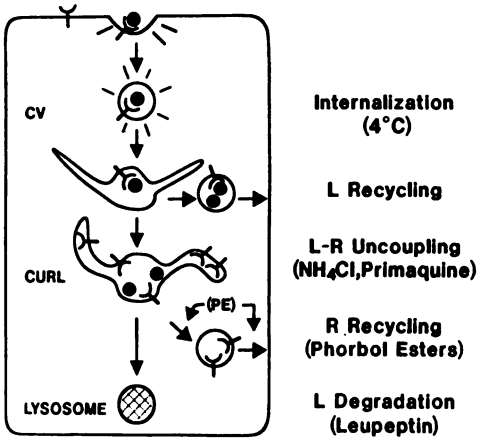

Fig. 6. Scheme of ligand and receptor pathways during receptor-mediated endocytosis of asialoglycoproteins. See text for discussion. PE, phorbol ester; CV, coated vesicle; L, ligand; R, receptor; *closed circles*, ligand molecules.

first hour of exposure to the agent (12). The internalized receptor molecules are not degraded but reside in an as yet uncharacterized intracellular location, from which they can readily return to normal distribution upon removal of the phorbols. Studies of this new steady state revealed that receptor recycling, with ligand uptake and delivery to lysosomes for degradation, still occurs although at a reduced rate relative to untreated controls (Table 1). An inhibition in the return of receptor to the plasma membrane as shown in Fig. 5 would be expected to produce such an effect on ASOR endocytosis, because the block is rapid in onset yet incomplete, permitting one-half of internalized receptors to reach the plasma membrane. Furthermore, the phorbol esters caused an immediate block in this pathway of ASGP receptor recycling to plasma membrane in primaquine-pretreated cells. However, in untreated cells with receptor molecules randomly distributed throughout the pathways of biosynthesis, degradation, and recycling, a considerably longer time would be required to reach a new steady state after exposure to phorbol esters. Indeed our previous studies suggested a decrease in surface receptor number beginning at 20 min and complete by 60 min (12).

Several other receptors participate in the pathways of internalization and recycling described above for the ASGP receptor. The transferrin receptor in K562 or HL60 cells (14, 24) and the low density lipoprotein receptor in U937 cells (25) similarly accumulate within the cell after exposure to phorbol esters. In contrast, receptors with low rates of recycling may not be redistributed following phorbol ester addition. For example, Iacopetta *et al.* (26) have shown in the HL60 cell line that unoccupied insulin receptors (low recycling rate) are not sequestered intracellularly when phorbols are added, although transferrin receptors in the same cell line (higher recycling rate) are redistributed from the plasma membrane to an intracellular location. These reports are consistent with the data in this paper and with the hypothesis that the major inhibitory site of phorbol esters on receptor-mediated endocytosis is located at the site of receptor recycling to plasma membrane. Recent morphological studies of the distal portions of receptor pathways within the cell demonstrate many potential targets for phorbol action. For example, uncoupled receptor molecules become clustered along the arms of the tubulovesicular structure known as CURL (3). Current quantitative data support the notion that this is an active process (6). From these tubular structures vesicles form for transport to the plasma membrane. The limitations of current whole cell physiological studies and the lack of pure subcellular fractions that can be assayed directly for these individual reactions make it impossible at present to precisely define the ultimate biochemical target of the phorbol esters. Figure 4 shows that ligand uncoupling in the presence of phorbol esters proceeds normally, in contrast to the effect seen with primaquine. This suggests that the phorbol ester block is located distal to the effects of lysosomotropic agents on vesicle acidification and ligand-receptor uncoupling. Despite their well-described actions of binding to and activation of protein kinase C in both whole cells and particulate fractions, it is premature to speculate whether the observed increases in receptor phosphorylation (reviewed in the introduction) are directly related to the intracellular redistribution seen after addition of these agents. It remains possible that phorbol esters act either by stimulating direct phosphorylation of other nonreceptor targets or by changes in the intracellular ionic milieu.

Another interesting feature of the block in the receptor recycling pathway described above is its incomplete nature. We have demonstrated that this occurs despite maximal phorbol ester concentrations and that it remains incomplete during our period of observation (2 hr). There are at least two circumstances that could lead to such a finding. One possibility is that phorbol esters affect a critical element of the trafficking/transport mechanism but can only produce 50% inhibition of this process. Another possibility would be that the pathway of receptor return to the plasma membrane is not uniform but is comprised of multiple parallel routes. The particular route followed by a single receptor molecule may then be determined by factors such as its location within CURL when its ligand dissociation is complete, the size or composition of the resultant transport vesicle, or the state of receptor phosphorylation or oligomerization. One or more of these routes might be blocked by phorbol esters to a nearly complete extent, resulting in receptor shunting to other routes or accumulation of intracellular receptor molecules within such a "dead-end" compartment. Further experiments on the ultrastructural location of phorbol ester-sequestered receptors are in progress and together with isolation of pure subcellular fractions and *in vitro* assays will allow a clearer definition of the mechanisms responsible for intracellular receptor movement. Phorbol esters undoubtedly will be of considerable usefulness in understanding these reactions and the role of structural accessory molecules involved in transporting receptors through the cell.

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