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Phorbol esters induce transient internalization without degradation of unoccupied epidermal growth factor receptors

(KB cells/protein kinase C/transreticular Golgi/recycling/phosphorylation)

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ABSTRACT **4β-Phorbol 12-myristate 13-acetate (PMA) treatment of KB cells at 37°C rapidly induces a 50% reduction in epidermal growth factor (EGF) binding that is maximal by 30 min. EGF binding activity returns to the original value by 1 hr and remains constant for 2 hr thereafter. Using a polyclonal antibody directed against the cytoplasmic domain of the EGF receptor (EGF-R), we examined the fate of the receptor after PMA treatment. Immunofluorescent and electron microscopic localization of the EGF-R after PMA treatment demonstrated that about 50% of the receptor became internalized into endocytic vesicles (receptosomes) and Golgi-associated structures. Unlike EGF-induced internalization, PMA-induced internalization did not cause delivery of EGF-R to lysosomes or receptor degradation. Rather, receptor reappeared on the cell surface. No stimulation of EGF-R synthesis was observed after 1 hr of PMA treatment. Loss of cell surface binding correlated with the internalization of the EGF-R observed morphologically. A possible explanation for these observations is that PMA, an activator of protein kinase C, confers a signal sufficient for EGF-R clustering and internalization but not for transport to lysosomes.**

Epidermal growth factor (EGF) and its receptor are internalized by the process of receptor-mediated endocytosis (1–4). EGF internalization was studied in the human epidermoid carcinoma cell line KB, because this line has a number of features that facilitate detailed studies of the endocytosis of EGF and its receptor. In KB cells the entry of EGF and its receptor is rapid and synchronous, the morphology of the cells is uniform, and there are sufficient receptors (≈200,000 per cell) to make detailed biochemical and morphological studies feasible. Upon EGF addition, the EGF receptor (EGF-R) rapidly clusters in coated pits at the cell surface. It is next found in endocytic vesicles and then associated with tubular elements lying on the trans side of the Golgi stacks (TR Golgi) and concentrated in the coated pits of the TR Golgi (5). Finally, the EGF-R is delivered to lysosomes, where it is degraded together with its ligand (5, 6). In cell culture, no recycling of the receptor has been described (7, 8, 42), although the kinetics of entry varies, according to the cell type studied (6, 9, 10). The physiological consequences of receptor internalization are still unclear, but it is of interest that other growth factor receptors (platelet-derived growth factor, insulin) share the same fate.

It would be of great interest to be able to modulate receptor internalization by other agents that could affect the fate of the receptor. Phorbol esters have been found to have a multiplicity of effects at the cell surface. Phorbol esters have been shown to affect the binding of insulin (11), somatomedin C (12), and EGF (13, 14); both an alteration in affinity and a decrease in total number of receptors have been reported (13, 15, 16). In the erythroleukemic cell line, K262, 4β-phorbol 12-myristate 13-acetate (PMA) has been found to stimulate internalization of the transferrin receptor in the absence of its ligand (17), whereas, in macrophages, an increase in plasma membrane transferrin receptors after treatment with phorbol esters has been reported (18). PMA has been found to stimulate the phosphorylation of the receptors for insulin, somatomedin C (19), and interleukin 2 (20), presumably by activating Ca^{2+}/phospholipid-dependent protein kinase C (21, 22).

The EGF-R is also a substrate for protein kinase C. Phosphorylation occurs on serine and threonine residues (23–25) and results in subsequent inhibition of EGF-stimulated tyrosine autophosphorylation (23, 26). Based on the amino acid sequence of the EGF-R (27, 28), Hunter *et al.* have shown that the major site of phosphorylation induced by protein kinase C is at threonine-654 (29). This residue is located in the intracellular portion of the receptor, nine residues from the proposed hydrophobic transmembrane domain.

Using PMA, the most potent of phorbol ester derivatives, we show here that the decrease in surface binding of EGF in KB cells (16) is due, at least in part, to internalization of the EGF-R. The process is rapid and transient, and the internalized EGF-R is not delivered to lysosomes for degradation. Instead, after ≈1 hr, EGF binding activity returns to the cell surface and may represent receptor recycling. These data suggest that the effect of PMA on the EGF-R may be a signal necessary and sufficient for internalization but not sufficient for directing the receptor to lysosomes, where it is degraded.

MATERIALS AND METHODS

Materials. PMA, phorbol 12,13-dibutyrate, and 4β-phorbol (Sigma) were dissolved in dimethyl sulfoxide at 10 mM and stock solutions were diluted to the appropriate concentration in Dulbecco's modified Eagle's medium (DMEM) prior to use. Mouse EGF (Bethesda Research Laboratories) was radioiodinated to a specific activity of 150–170 μCi/μg (1 Ci = 37 GBq) by the chloramine-T method (30). [^{35}S]Methionine (1100 Ci/mmol) was from Amersham and ^{14}C-labeled molecular weight standards were from New England Nuclear. EGF-R antiserum was obtained by immunizing a rabbit with purified human EGF-R (31). For immunofluorescence and immunocytochemistry experiments, the antibody was affinity-purified on an EGF-Affi-Gel column (32) to which the purified human EGF-R was covalently coupled (unpublished data). The antibody recognizes an intracellular determinant of the receptor (unpublished data).

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Abbreviations: EGF, epidermal growth factor; EGF-R, EGF receptor; PMA, 4β-phorbol 12-myristate 13-acetate; TR Golgi, transreticular Golgi.

Binding of ^{125}I-Labeled EGF (^{125}I-EGF). KB cells, grown as described (5), were planted, if not otherwise stated, in 35-mm plastic tissue culture dishes at 5×10^5 cells per dish (80% confluent). To measure EGF binding, the monolayers were washed twice and incubated in serum-free DMEM/20 mM Hepes, pH 7.5, for 1 hr at 37°C and then incubated for the indicated time with PMA, 4β-phorbol, or EGF. Cells were then washed with ice-cold medium and incubated for 2 hr at 4°C in 2 ml of binding medium (DMEM/20 mM Hepes, pH 7.5/0.1% bovine serum albumin) containing ^{125}I-EGF (2×10^5 cpm/ng) at a final concentration of 1.7 nM. Nonspecific binding, estimated in the presence of 1 μg of unlabeled EGF per ml, was <1.5%. After the incubation, monolayers were washed four times with ice-cold binding medium, solubilized with 1 ml of 1 M NaOH, transferred to plastic test tubes, and assayed for radioactivity in a γ-counter.

Metabolic Labeling and Immunoprecipitation. KB cells were seeded at a density of 2.5×10^6 cells per 60-mm tissue culture dish. Eight to 10 hr later the cultures were radiolabeled for ≈12 hr with [^{35}S]methionine (250 μCi/ml) in 2 ml of methionine-free Eagle's medium with 5% fetal bovine serum. Cells were then washed and preincubated for 1 hr in serum-free medium before treatment with 4β-phorbol, PMA, or EGF. The cells were then harvested and lysed as described (6). Cell extracts were clarified at 100,000 × *g* for 30 min and preabsorbed with 0.2 ml of formalin-fixed *Staphylococcus aureus* (10%, wt/vol) for 30 min at 4°C. For immunoprecipitation, equal amounts of [^{35}S]methionine-labeled extracts were precipitated with 20 μl of anti-EGF-R antisera followed by 50 μl of *S. aureus* (33). The immunoprecipitated proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (PAGE) on 8.5% gels (34). Gels were fluorographed (35) and autoradiographed. Quantitation was achieved by excising the [^{35}S]methionine EGF-R protein band; gel slices were extracted with 2 ml of 30% H_2O_2/1% NH_4OH for 24 hr at 37°C and radioactivity was counted in 20 ml of Aquasol. [^{35}S]Methionine incorporation into total cell protein was determined by precipitating duplicate aliquots of each cell extract with 20% trichloroacetic acid using 50–100 μg of bovine serum albumin as a carrier.

Immunofluorescence and Electron Microscopic Immunocytochemistry. Subconfluent KB cells in 35-mm tissue culture dishes were preincubated for 1 hr at 37°C in serum-free medium and then exposed to PMA, 4β-phorbol, phorbol 12,13-dibutyrate, or EGF for different times. Cells were then fixed and permeabilized as described (6); they were sequentially incubated with affinity-purified anti-EGF-R antibody at 50 μg/ml followed by affinity-purified rodamine goat anti-rabbit IgG (Jackson ImmunoResearch, Avondale, PA) at 50 μg/ml. Antibody incubations were for 15 min at 23°C. Controls included substitution of normal rabbit IgG or deletion of the first step. Cells were then processed as described (6). Parallel dishes were used for electron microscopic immunocytochemistry. Cells were fixed and processed by using the "EGS" procedure with 0.1% glutaraldehyde (36). The EGF-R was localized by using affinity-purified anti-EGF-R antibody (50 μg/ml) and the ferritin bridge method (36). Cells were post-fixed, dehydrated, and embedded as described (36). Thin sections were counterstained with lead citrate and bismuth subnitrate and examined at 40 kV.

RESULTS

KB cells were incubated with 100 nM EGF, 100 nM PMA, or 100 nM 4β-phorbol for 5–180 min and then their EGF binding capacity was measured. Consistent with previous reports (6, 8), when cells were treated with 100 nM EGF, their binding capacity rapidly fell to very low levels and remained low for 180 min (Fig. 1). When treated with PMA, EGF binding decreased rapidly over the initial 10 min, reached about 50% of the starting value at 30 min, and remained depressed until 40–50 min. After about 1 hr, EGF binding had returned to the initial value. 4β-Phorbol, an analogue incapable of activating protein kinase C, had no effect on EGF binding. When the concentration-dependent inhibition of EGF binding by PMA was analyzed, a small effect was noted at 10 nM PMA, with an increasing response up to 1 μM (Table 1). Even at this high concentration of PMA, only about 50% of the receptor was found to be internalized after the 40-min point. In the studies shown in Fig. 1 and Table 1, EGF binding was measured by using 1.7 nM EGF (10 ng/ml), a level that is slightly higher than the K_d reported for EGF binding on KB cells (8, 16). This result indicates that PMA decreased the ability of KB cells to bind EGF by inducing a change in either binding capacity or affinity.

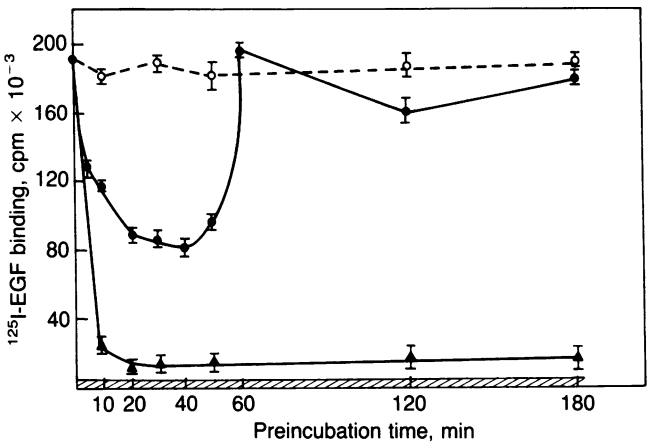

FIG. 1. Binding of ^{125}I-EGF to the surface of KB cells after preincubation with PMA, 4β-phorbol, or EGF. Cells were incubated for 1 hr in serum-free DMEM. Unlabeled EGF (▲), PMA (●), or 4β-phorbol (○) was then added to a final concentration of 100 nM and the incubation at 37°C was continued for up to 3 hr. The monolayers were then washed twice with ice-cold medium and incubated for 2 hr with ^{125}I-EGF (2×10^6 cpm) on ice. Cells were then rinsed four times and their content of ^{125}I-EGF was determined after solubilization with 1 M NaOH.

To determine if the change in EGF binding reflected a change in binding site number, affinity, or both, EGF binding was measured before PMA addition, 30 min after PMA addition (when EGF binding had fallen by 50–60%), and 90 min after EGF addition (when the level of EGF binding had returned to approximately normal values). The data of Fig. 2 indicate that the decrease in EGF binding observed at 30 min was not accompanied by a detectable change in apparent affinity. Half-maximal binding of EGF occurs at 1.5 nM EGF without PMA treatment, at 1.2 nM EGF 30 min after PMA

Table 1. PMA-induced inhibition of ^{125}I-EGF binding: Concentration dependence

Phorbol	% of control EGF binding
4β-Phorbol, 1000 nM	102
PMA, nM	
1	100
10	84
50	72
100	66
500	48
1000	44

KB cells were preincubated for 1 hr in serum-free DMEM with the indicated concentration of PMA or 4β-phorbol. Then cells were cooled to 4°C and the binding of ^{125}I-EGF to the monolayers was measured.

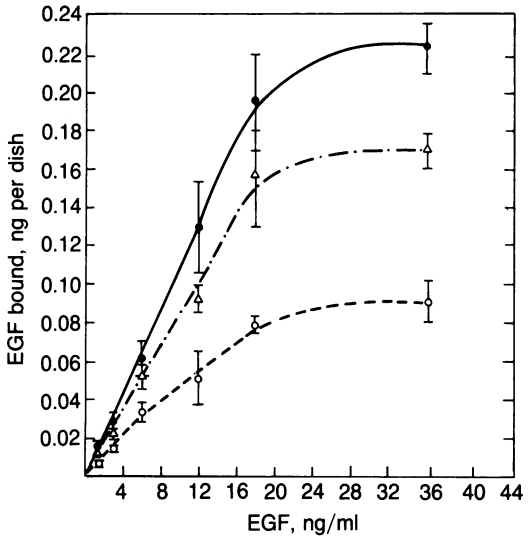

FIG. 2. Concentration dependence of ^{125}I-EGF binding to KB cells after PMA pretreatment. Cells seeded in 24-multiwell dishes at 1.5×10^5 cells per dish (80% confluence) were washed twice and preincubated for 1 hr at 37°C in serum-free medium. The cells were then incubated with 100 nM PMA for 30 min (○) or 90 min (△) or with no pretreatment at all (●). The monolayers were washed and then cooled at 4°C, and the binding of EGF was performed for 2 hr at 4°C; subsequently, the cells were treated as reported in the legend of Fig. 1. The concentration of ^{125}I-EGF was varied from 1.5 to 150 ng/ml by increasing the amount of labeled or unlabeled EGF. Nonspecific binding, ranging between 1% and 5%, was measured at a concentration of EGF of 1 μg/ml and was subtracted from reported values.

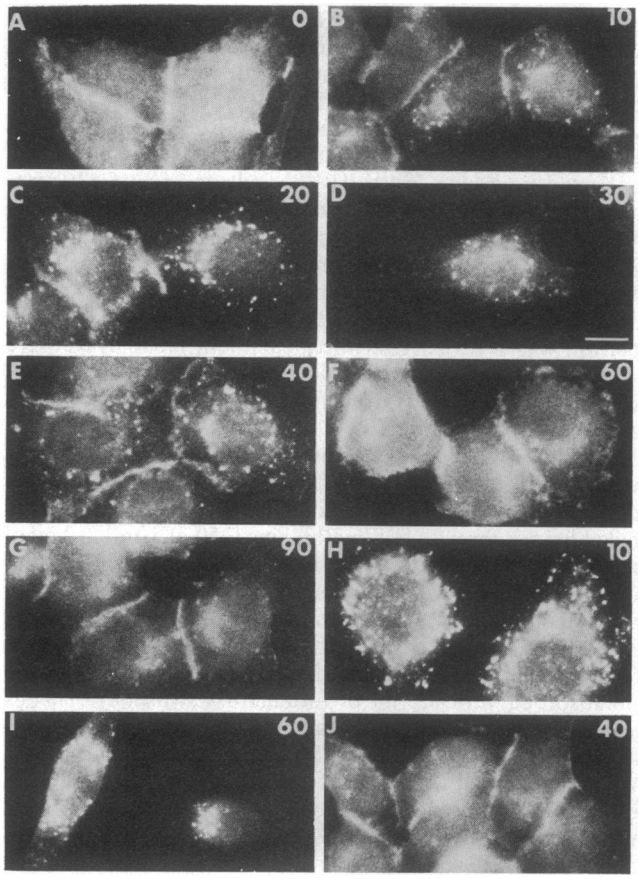

FIG. 3. Immunofluorescence localization of the EGF-R in KB cells after incubation with PMA, EGF, or 4β-phorbol. KB cells were preincubated in serum-free DMEM for 1 hr at 37°C and then incubated at 37°C in the presence of 100 nM PMA for 10 (*B*), 20 (*C*), 30 (*D*), 40 (*E*), 60 (*F*), and 90 (*G*) min, in the presence of 100 nM EGF for 10 (*H*) and 60 min (*I*), or in the presence of 100 nM 4β-phorbol for 40 min (*J*) or immediately fixed (*A*). After fixation in formaldehyde and permeabilization with Triton X-100, the EGF-R was localized. The receptor can be seen diffusely distributed on the cell surface in the absence of any ligand (*A*) or after preincubation for 40 min with 4β-phorbol (*J*); the receptor is completely internalized after EGF addition in a punctuate pattern of receptosome-like (10 min, *H*) and lysosome-like (60 min, *I*) vesicles. After PMA incubation ranging from 10 to 40 min (*B–E*), a similar punctuate pattern can be visualized, reaching a maximum at 20–30 min. Note that at any time after PMA pretreatment, there is still immunoreactive EGF-R at the cell surface. At 60 and 90 min after PMA addition (*F* and *G*) the receptor can be localized again, mostly at the cell surface. (×580; bar = 10 μm.)

treatment, and at 1.4 nM EGF 90 min after PMA treatment. These data argue that the major effect of PMA on KB cells is to lower the number of EGF-Rs without altering their affinity for the ligand.

A possible explanation for a decrease in binding capacity is internalization of surface receptors without reinsertion of new receptors at the cell surface. To determine if PMA induces internalization of the receptor, cells were fixed at various times after treatment with 100 nM PMA and the receptor location was established by using a rabbit anti-EGF-R antibody (Fig. 3).

Before PMA addition, the receptor exhibited a typical diffuse plasma membrane distribution (Fig. 3*A*). As early as 10 min after PMA treatment, the receptor distribution had begun to change. Some receptors remained on the surface but the rest appeared as small bright spots within the cell. This distribution is characteristic of the receptor being located in intracellular endocytic vesicles (receptosomes). The vesicular pattern reached a maximum of intensity between 20 and 40 min (Fig. 3 *C–E*), but at all times significant amounts of the receptor remained on the surface. After 60 min and at later time points, the intracellular vesicular distribution completely disappeared, and the receptor was found again associated with the plasma membrane (Fig. 3 *F* and *G*). Thus, the decrease in surface binding, detected between 10 and 40 min, was accounted for by movement of the receptor away from the cell surface into intracellular vesicular elements. The return of the EGF-R to the surface was not due to exhaustion or inactivation of the PMA because medium, removed from cells treated with 100 nM PMA for 1 or 2 hr, induced a normal response (i.e., internalization) when added to cells not previously exposed to PMA (data not shown). Another active phorbol derivative, 4β-phorbol 12,13-dibutyrate, was also found to induce EGF-R internalization with this fluorescence assay (data not shown).

In a parallel control experiment, 100 nM EGF was added for 10 and 60 min. At 10 min (Fig. 3*H*), EGF was found in vesicles randomly distributed in the cell with the distribution of receptosomes, as also observed after PMA treatment. At 60 min (Fig. 3*I*), as expected from previous results (6), the receptor was localized in perinuclear vesicles with the appearance of lysosomes (6). No effect on receptor distribution was detected after treatment with 4β-phorbol (Fig. 3*J*).

Electron Microscopic Studies. To determine the precise intracellular location of the EGF-R after PMA treatment, electron microscopic immunocytochemistry was performed (Fig. 4). The EGF-R was found concentrated in receptosomes at 10 (Fig. 4*A*) and 35 min (Fig. 4*B*) after PMA addition. Ferritin cores that are attached to the antibody were observed on the cytoplasmic surface of the vesicles, because the antibody recognizes an intracellular determinant of the EGF-R (unpublished data). A large amount of the receptor was still detected on the plasma membrane at 10 and 35 min (Fig. 4*E*). At 35 min, the receptor was also localized in tubular and vesicular profiles near the margin of endocytic vesicles; some of these tubular profiles were closely associated with

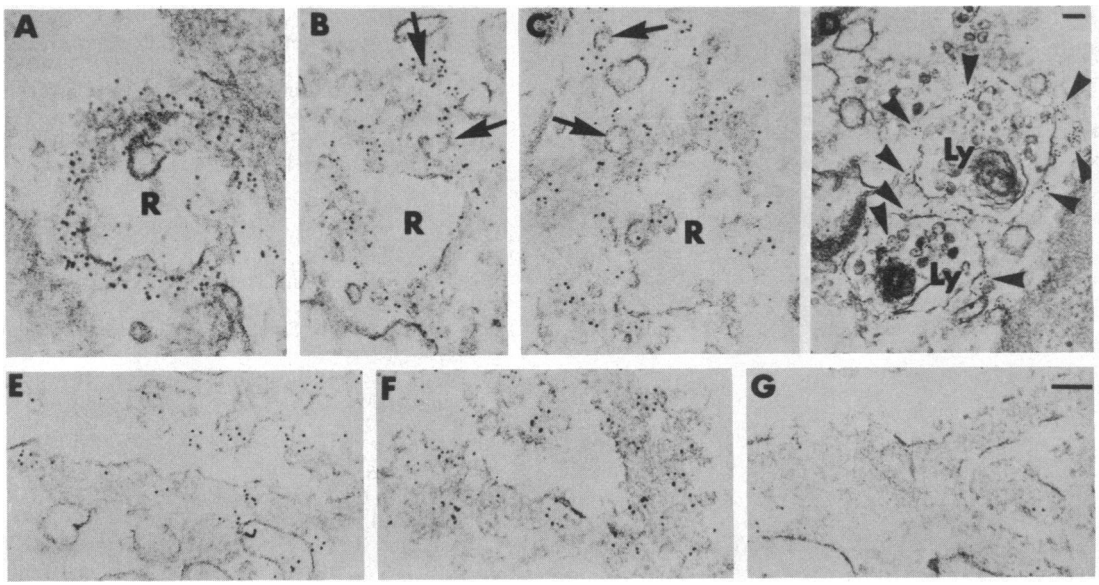

FIG. 4. Electron microscopic immunocytochemical localization of the EGF-R. KB cells preincubated for 1 hr in serum-free medium were treated with PMA (*A–C, E* and *F*) or EGF (*D* and *G*) for 10 (*A*), 35 (*B–E* and *G*), or 60 (*F*) min. The cells were then fixed and processed by using the EGS and ferritin bridge procedure and anti-EGF-R antibody. The receptor was found concentrated in receptosomes (R) at 10 min after PMA treatment (*A*) and in receptosomes and adjacent tubular elements (arrows) at 35 min after PMA treatment (*B* and *C*). A considerable amount of receptor could still be found on the plasma membrane (*E*). At 60 min after PMA treatment, large amounts of receptor were present on the plasma membrane (*F*), but only small amounts were found in lysosomes. In contrast, EGF treatment for 35 min caused large amounts of receptor (arrowheads) to accumulate in lysosomes (Ly) (*D*). At the same time, very little receptor was still present on the cell surface (*G*). (*A–C* and *E–G*, ×39,000; *D*, ×20,000; bar = 0.1 μm.)

clathrin-coated regions (Figs. 4 *B* and *C*), but no concentration of ferritin was detected within the clathrin-coated profiles. At 35 min, no significant amount of receptor was found in lysosomes. In contrast, cells treated with EGF for 35 min accumulated large amounts of receptor in lysosomes (Fig. 4*D*), with very little receptor left on the cell surface (Fig. 4*G*). Sixty minutes after PMA treatment, much of the intracellular receptor had disappeared and a high concentration of receptor was found again on the plasma membrane (Fig. 4*F*).

Metabolic Labeling Studies. It was previously shown that addition of EGF to KB cells results in degradation of the EGF-R with a half-life of ≈50 min (ref. 6 and Fig. 5). To examine any possible effects of PMA on receptor turnover, cells previously labeled for 16 hr with [^{35}S]methionine were exposed to PMA. As shown in Fig. 5, PMA did not promote the degradation of prelabeled receptor. In fact, the amount of [^{35}S]methionine found in the EGF-R in PMA-treated cells rose slightly, suggesting that either PMA was selectively inhibiting its breakdown or effectively promoting reincorporation of [^{35}S]methionine into the receptor.

To assess whether PMA affects receptor synthesis, cells were labeled for 1 hr with [^{35}S]methionine at 250 μCi/ml in the presence of either PMA or 4β-phorbol and the EGF-R was immunoprecipitated and isolated by NaDodSO$_4$/PAGE as described above. The results showed that PMA did not increase the incorporation of [^{35}S]methionine into the EGF-R; 3040 cpm were detected in the EGF-R band with 4β-phorbol and 2740 cpm with PMA.

DISCUSSION

The results in this paper provide evidence that KB cells treated with 100 nM PMA, in the absence of EGF, transiently internalize ≈50% of the cell surface EGF-Rs. The ability of the cells to bind EGF decreases rapidly after PMA treatment but is nearly restored quantitatively after 1 hr, even in the continuous presence of PMA. This PMA-induced internalization, unlike EGF-induced uptake, does not result in substantial receptor delivery to lysosomes or degradation. Taken together, these findings suggest that PMA promotes internalization of the EGF-R either by directly stimulating a modification of the receptor or by modifying another component involved in receptor internalization (for example, a component of coated pits). In response to phorbol ester treatment, EGF-R has been recently shown to be phosphorylated on a threonine residue that lies close to the cytosolic face of the plasma membrane (29). These data suggest that PMA-induced phosphorylation of a specific threonine site by protein kinase C is directly involved in inducing EGF-R internalization.

Although both EGF and PMA cause the receptor to be internalized into coated pits and endocytic vesicles, the ultimate fate of the internalized receptor is clearly different. With EGF, nearly all of the EGF-R is removed from the cell surface in a few minutes. With PMA, about half of the receptor and associated binding activity remains on the surface at the time of maximal PMA response. What is the explanation for this partial response to PMA? It is not due to an insufficient amount of PMA, because even at high levels of PMA (up to 10 μM) only a partial effect is observed. It is possible that the rate of EGF-R internalization produced by PMA is slow and asynchronous so that, while a subset of receptors is inside, newly entered molecules replace those returning to the cell surface. It is also possible that there are two classes of EGF-Rs and only one class is capable of responding to PMA. If protein kinase C is involved, this molecule may be limiting unless "recruited" to the cell surface by another mechanism (22, 37). Ca^{2+} has been suggested to be involved in association of normally soluble protein kinase C with plasma membranes (37, 38). In addition, Ca^{2+} has been shown to act synergistically in altering EGF internalization (39, 40).

By using antibodies to the EGF-R it was possible to determine the location of the EGF-R internalized in the presence of PMA and to compare this location with that induced by EGF. EGF induces its receptor to associate in succession with the following organelles: coated pits, receptosomes, TR Golgi elements, and, finally, lysosomes, where the receptor is degraded. This morphological pathway

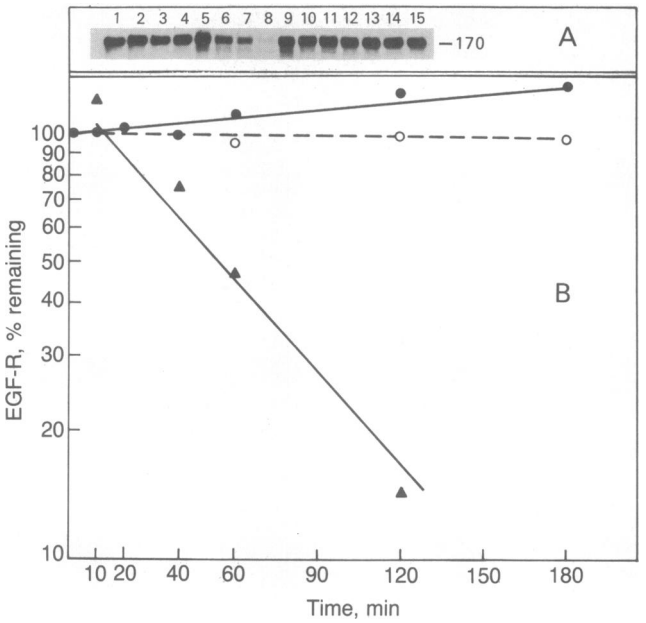

FIG. 5. (*A*) Immunoprecipitation of [^{35}S]methionine-labeled EGF-R. [^{35}S]Methionine-labeled cells were preincubated in serum-free medium for 1 hr at 37°C and then lysed immediately (lane 1) or incubated for 10 (lanes 5 and 9), 20 (lane 10), 40 (lanes 6, 11, and 14), 60 (lanes 2, 7, and 12), 120 (lanes 3, 8, and 13), and 180 min (lanes 4 and 14) in the presence of 100 nM PMA (lanes 9–14), 100 nM EGF (lanes 5–8), 100 nM 4β-phorbol (lane 15), or control medium (lanes 1–4). For each lane, 5×10^7 cpm of [^{35}S]methionine-labeled extracts, determined by precipitation with trichloroacetic acid, was immunoprecipitated with anti-EGF-R antibody antiserum. Exposure was for 12 hr. (*B*) Quantitation of the half-life of EGF-R immunoprecipitated after *in vivo* exposure to PMA or EGF. The EGF-R bands shown in *A* were excised and the radioactivity was counted. The percentage of cpm in the residual EGF-R immunoprecipitated was plotted as a function of the time of incubation with PMA (●), EGF (▲), or control medium (○).

is only in part shared by EGF-R internalized in response to PMA. PMA induces internalization by stimulating clustering of the receptor in coated pits (unpublished data), transfer to receptosomes, and, next, transfer to elements of the TR Golgi. Unlike the EGF-induced response, EGF-R is not found in lysosomes. The failure to detect EGF-R in lysosomes is in agreement with the observation that the EGF-R is not degraded in PMA treated cells (Fig. 5 and ref. 10). Also, different from the EGF-induced response is the reappearance of the receptor on the cell surface. The receptor that reappears on the cell surface is probaby the receptor induced to be internalized by PMA and not newly synthesized receptor because PMA had no effect on EGF-R synthesis when measured 1 hr after PMA treatment. Also, the fact that the receptor binding activity recovers so rapidly after internalization makes *de novo* synthesis unlikely. If our suggestion that PMA causes the EGF-R to be internalized and recycled is correct, the morphological pathway would closely resemble that previously observed for the transferrin receptor (41). This would indicate that PMA provides only a signal for internalization and not for lysosomal delivery.

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