Role of Intracellular Calcium and Protein Kinase C in the Endocytosis of Transferrin and Insulin by HL60 Cells

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Abstract. The role of the cytosolic free calcium concentration ($[Ca^{2+}]_i$) and of protein kinase C on the internalization of transferrin and insulin in the human promyelocytic cell line HL60 was investigated. $[Ca^{2+}]_i$ was selectively monitored and manipulated by the use of the fluorescent Ca^{2+} indicator and buffer quin2, while receptor–ligand internalization was studied directly by quantitative electron microscope autoradiography. Decreasing the $[Ca^{2+}]_i$ up to 10-fold below resting level had no effect on the internalization of transferrin or insulin. Similarly, a 10-fold elevation of the $[Ca^{2+}]_i$ using the calcium ionophore ionomycin caused little or no change in the endocytosis of the two ligands. In contrast, activation of protein kinase C by phorbol myristate acetate markedly stimulated the internalization of both occupied and unoccupied transferrin receptors, even in cells with very low $[Ca^{2+}]_i$. The insulin receptor was found to behave differently in response to phorbol myristate acetate, however, in that only the occupied receptors were stimulated to internalize. We conclude that the $[Ca^{2+}]_i$ plays only a minor role in regulating receptor-mediated endocytosis, whereas protein kinase C can selectively modulate receptor internalization depending on receptor type and occupancy.

Upon binding to cell surface receptors, both transferrin (14, 37) and insulin (3) are internalized by receptor-mediated endocytosis. The signals provided for the aggregation of receptors or receptor–ligand complexes into coated pits at the cell surface and for their subsequent internalization and recycling are unknown. Two possible intracellular candidates for the modulation of receptor movement are the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$)[1] and the ubiquitous enzyme protein kinase C. Activation of protein kinase C by phorbol esters (6) has been found to stimulate the internalization of both occupied and unoccupied transferrin receptors (11, 18, 23). For the role of $[Ca^{2+}]_i$, it has been suggested that localized Ca^{2+} gradients are involved in generating the signals for phagocytosis (21, 40) and that Ca^{2+}-regulated processes could be involved in endocytosis (11, 30). Furthermore, increasing intracellular $[Ca^{2+}]_i$ seems to result in an inhibition of internalization of surface-bound ligands, e.g., insulin growth factor II and epidermal growth factor (19, 22, 25). Recently the development of the tetracarboxylate Ca^{2+} indicator and chelator quin2 (34–36) has permitted the modulation and measurement of $[Ca^{2+}]_i$ in small mammalian cells. One of the major biological applications of quin2 has been in the study of the role of $[Ca^{2+}]_i$ in exocytosis; as far as we are aware, however, this technique has not previously been used to study the reverse process, i.e., endocytosis.

In the present study we have investigated the roles of $[Ca^{2+}]_i$ and protein kinase C in receptor turnover by using quin2 and Ca^{2+} ionophores (ionomycin, A23187) to respectively decrease and increase the $[Ca^{2+}]_i$, and by using phorbol myristate acetate (PMA) to activate protein kinase C. We have then tested the influence of these various parameters on the receptor-mediated endocytosis of two diverse classes of ligands: transferrin, representing the nutrient uptake system, and insulin, representing the signal peptide system. The results indicate that internalization of transferrin and insulin receptors is relatively insensitive to the $[Ca^{2+}]_i$, while activation of protein kinase C can selectively modulate receptor internalization.

1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, cytosolic free calcium concentration; DMSO, dimethyl sulfoxide; ^{125}I-tn, radioiodinated transferrin; PMA, phorbol myristate acetate.

Materials and Methods

Cells and Reagents

HL60 cells were grown in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) and 10 mM Hepes buffer, pH 7.2. Cells were used when in the logarithmic phase of growth. Human apotransferrin was purchased from Sigma Chemical Co. (St. Louis, MO) and converted to ferrotransferrin by incubation for 30 min at room temperature with 2 mol $FeCl_2$ per mole of transferrin. 100 μg of ferrotransferrin

was radioiodinated with 1 mCi of ^{125}I using Enzymobeads (Bio-Rad Laboratories, Richmond CA) according to the manufacturer's instructions. Radioiodinated transferrin (^{125}I-tn) was separated from free ^{125}I using Sephadex G-25 gel chromatography. Human apotransferrin was also labeled with ^{59}Fe as previously described (15). Radioiodinated A14 pork insulin (gift from Eli Lilly & Co., Indianapolis, IN) was prepared and purified by high-performance liquid chromatography before lyophilization for shipment. The specific activities of the radiolabeled ligands were 4–6 μCi/μg for ^{125}I-tn and 250–350 μCi/μg for ^{125}I-insulin. PMA (Sigma Chemical Co.) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 μM and a stock solution stored at −20°C. A 1:2,000 dilution of this stock solution was used to give a final concentration of 50 nM used in the experiments. The calcium ionophores ionomycin (gift from Dr. Liu of Hoffman-la-Roche, Nutley, NJ) and A23187 (Sigma Chemical Co.) were dissolved in DMSO. Quin2 acetoxy-methylester (quin2) and quin2 free acid were purchased from Amersham Corp. (Arlington Heights, IL) and Sigma Chemical Co.

Measurement of $[Ca^{2+}]_i$

Quin2 loading was performed essentially as described previously (20, 27). HL60 cells from the logarithmic growth phase were washed twice by centrifugation in RPMI and resuspended at a concentration of 5×10^7 cells/ml in medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM $NaHPO_4$, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes, pH 7.4. This medium is referred to as calcium medium ($[Ca^{2+}]_o = 10^{-3}$ M). In indicated experiments, calcium-free medium ($[Ca^{2+}]_o = 10^{-9}$ M) consisting of the above medium but without calcium and containing 1 mM EGTA was also used. After equilibration of the cells for 5 min at 37°C in either of these two media, quin2 was added from a 20 mM stock solution in DMSO to give a final concentration of 30–50 μM and the cells were incubated for 60 min at 37°C. 10 min after quin2 addition, the cells were diluted to 1×10^7 cells/ml with warm medium plus 0.5% BSA. Control cells were treated in parallel with 0.5% DMSO and then diluted as were the test cells. After loading, the cells were washed and resuspended in the same medium without albumin and kept at room temperature until used.

Fluorescence measurements of quin2 loaded and control cells were made in calcium or calcium-free medium at 37°C in a Perkin-Elmer fluorescence spectrophotometer (LS-3, Perkin Elmer Corp., Norwalk, CT) using an excitation wavelength of 339 ± 5 nm and an emission wavelength of 492 ± 10 nm. The cuvette was thermostated at 37°C and magnetically stirred. Intracellular quin2 concentrations were determined by comparing the Ca^{2+}-dependent fluorescence of quin2-loaded cells, which had been treated with 0.1% Triton, with the fluorescence of a standard solution of quin2 free acid in the presence of unloaded cells, which had been treated with 0.1% Triton in calcium medium (27, 36).

Cell Incubations

Quin2-loaded cells at resting $[Ca^{2+}]_i$ were obtained by loading with quin2 in calcium medium as described above. Cells with low $[Ca^{2+}]_i$ were obtained by loading with quin2 in calcium-free medium, while high $[Ca^{2+}]_i$ was obtained by incubation with either 1 μM ionomycin or 1 μM A23187 in calcium medium. In the experiments for autoradiography, cells were washed and resuspended in the same medium in which they had been loaded with quin2 (calcium or calcium-free), and incubated with radiolabeled ^{125}I-tn (5–10 μg/ml) or ^{125}I-insulin (5–25 ng/ml) for up to 30 min at 37°C in the presence or absence of calcium ionophores or PMA. At various times of incubation cells were washed with ice-cold PBS to remove unbound ligand and fixed for electron microscopy (see below).

The specificity of transferrin and insulin receptors in HL60 cells was examined by incubation with the respective radiolabeled ligands in the presence of a 200–1,000-fold excess unlabeled ligand and comparing the radioactive uptake to cells incubated with the radiolabeled ligand. Radioactivity associated with cells in the presence of excess unlabeled ligand was considered to be due to nonspecific binding and accounted for no more than 6% of the ^{125}I-tn binding and 3% of the ^{125}I-insulin binding.

Electron Microscope Autoradiography

At the end of the incubations with ^{125}I-tn or ^{125}I-insulin (see above), cells to be used for electron microscope autoradiography were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 30 min at room temperature. They were then washed several times in the cacodylate buffer and further processed for electron microscope autoradiography as previously described (3). The autoradiographic grains were analyzed quantitatively.

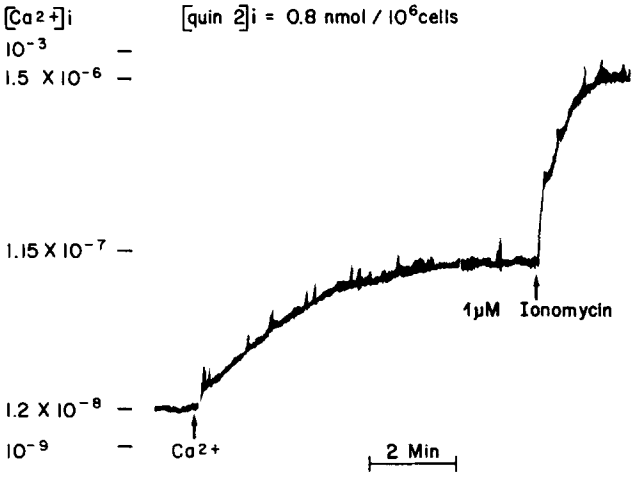

Figure 1. Depletion and elevation of $[Ca^{2+}]_i$ in HL60 cells by quin2 loading and addition of ionomycin. Cells were loaded with quin2 in calcium-free medium + EGTA as described in the Materials and Methods section. After loading, the cells were washed and resuspended in the same medium and the $[Ca^{2+}]_i$ determined by measurement of the quin2 fluorescence. The $[Ca^{2+}]_i$ calibration of the quin2 fluorescence is shown on the left side. Where indicated, 2 mM $CaCl_2$ and 1 μM ionomycin were added, giving rise to resting $[Ca^{2+}]_i$ (115 nM) and high $[Ca^{2+}]_i$ (≅1,500 nM), respectively. The intracellular quin2 concentration of loaded HL60 cells was in the range of 2–3 mM.

Under our assay conditions the H.D. (half-distance) is ~100 nm and the plasma membrane of the cells approximates a line source of irradiation (31). To analyze a large number of grains, we simulated this line source model. Autoradiographic grains representing the ^{125}I-tn or ^{125}I-insulin were classified at the electron microscope as originating from the cell surface if any portion of the grain overlapped the surface membrane. Grains that were contained completely within the cell and had no portion overlapping the cell surface were considered to present internally located ^{125}I-tn or ^{125}I-insulin. The results obtained using this procedure correlate to within 5% of those obtained with the circle technique used in previous studies (3). This variation made it possible to analyze a much larger number of grains than is practically possible by photographing each grain. At least 500 grains for ^{125}I-tn

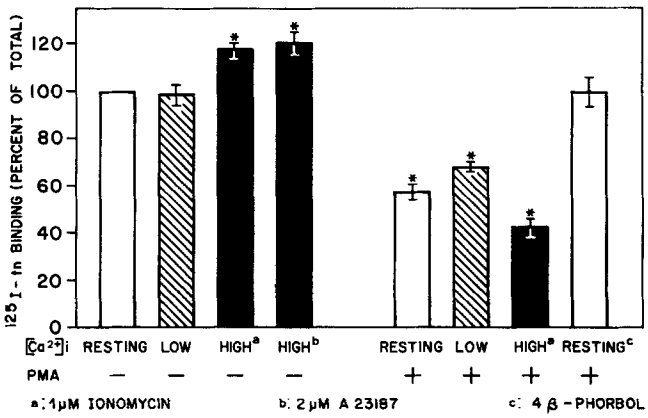

Figure 2. The effect of $[Ca^{2+}]_i$ and phorbol esters on the surface binding of ^{125}I-tn to HL60 cells. Cells in conditions of low, resting, and high $[Ca^{2+}]_i$, obtained as described in the Materials and Methods, were preincubated in the presence or absence of 50 nM PMA for 15 min at 37°C, washed, and then reincubated with ^{125}I-tn for 30 min at 4°C. Also examined was the phorbol analogue 4β-phorbol. Results shown are the surface binding of ^{125}I-tn expressed as a percentage of the control (mean ± SEM of three experiments). *, $P < 0.05$.

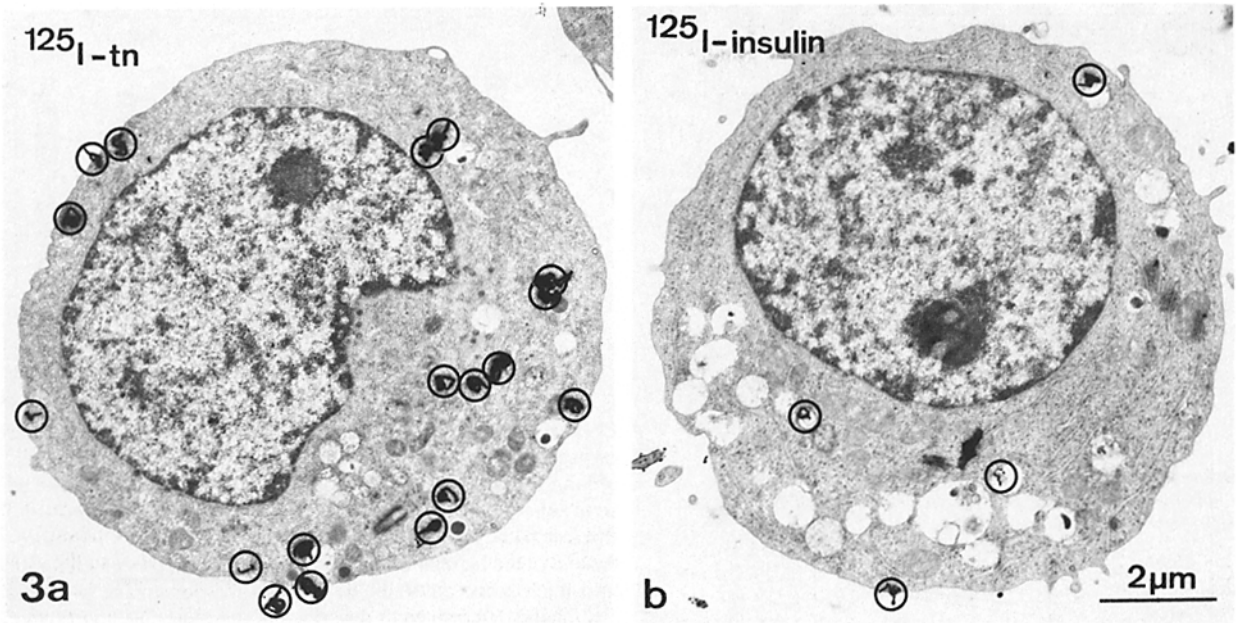

Figure 3. Thin sections of HL60 cells with developed autoradiographic grains. (*a*) Representative picture of a cell with resting $[Ca^{2+}]_i$ incubated with ^{125}I-tn for 15 min at 37°C, and (*b*) of a cell with resting $[Ca^{2+}]_i$ incubated with ^{125}I-insulin for 15 min at 37°C.

and 300 grains for ^{125}I-insulin were counted at the microscope for each condition studied in all experiments.

Results

Depletion and Elevation of $[Ca^{2+}]_i$ in HL60 Cells

By loading HL60 with quin2 in calcium-free medium, the $[Ca^{2+}]_i$ can be lowered from normal resting levels of 80–120 nM down to 10–15 nM, i.e., a 5–10-fold decrease (Fig. 1). Upon readdition of $CaCl_2$ to the medium the $[Ca^{2+}]_i$ returns to resting levels within ~5 min due to the influx of Ca^{2+} into the cell. Addition of 1 μM ionomycin to cells at resting $[Ca^{2+}]_i$ in calcium-containing medium results in a rapid and sustained 15–20-fold increase in the $[Ca^{2+}]_i$ (Fig. 1). Thus, by using the Ca^{2+} chelator quin2 and the Ca^{2+} ionophore ionomycin, the $[Ca^{2+}]_i$ could be respectively depleted or elevated to concentrations well outside the physiological range. In neither condition did the cell morphology appear to be altered in comparison to control cells.

Effects of $[Ca^{2+}]_i$ Depletion and Elevation on Transferrin and Insulin Binding

Preliminary experiments showed that the quin2 loading procedure, in either calcium medium (resting $[Ca^{2+}]_i$) or calcium-free medium (low $[Ca^{2+}]_i$), caused no significant changes in the surface binding of ^{125}I-tn (Fig. 2). However by elevating the $[Ca^{2+}]_i$ with either ionomycin or A23187, the surface binding of ^{125}I-tn was increased by 18 and 20%, respectively. This result agrees with recent observations by May et al. (24) in the same cell type. The surface binding of ^{125}I-insulin to cells in conditions of low or high $[Ca^{2+}]_i$ was not significantly different to that of unloaded cells or quin2-loaded cells at resting $[Ca^{2+}]_i$ (results not shown).

Effect of $[Ca^{2+}]_i$ Depletion and Elevation on Transferrin Endocytosis

To examine the effect of low and high $[Ca^{2+}]_i$ on transferrin endocytosis, cells from the two conditions were incubated at 37°C with ^{125}I-tn and the internalization at various times determined by electron microscope autoradiography as described in the Materials and Methods. Endocytosis by cells with resting $[Ca^{2+}]_i$ reaches a steady state in ~15 min, by which time ~60% of the total cell-bound ^{125}I-tn is internal (Figs. 3 and 4). Cells with depleted $[Ca^{2+}]_i$ could internalize ^{125}I-tn to the same extent as cells with resting $[Ca^{2+}]_i$ (Figs. 4 and 5), indicating that endocytosis can proceed normally at very low $[Ca^{2+}]_i$.

Since depletion of $[Ca^{2+}]_i$ to very low levels appeared to have no effect on the endocytosis of ^{125}I-tn, we next examined whether large increases in $[Ca^{2+}]_i$ induced by ionomycin could perturb the endocytotic process. At $[Ca^{2+}]_i$ 15–20-fold higher than resting level, a small (10–15%) but significant ($P < 0.05$, $n = 4$) reduction in the internalization of ^{125}I-tn was observed (Figs. 4 and 5). To obtain further information on this apparent inhibition of endocytosis, we measured the rate of ^{59}Fe accumulation from ^{59}Fe-tn in the presence of calcium ionophores. After a 60-min incubation at 37°C, the cellular uptake of ^{59}Fe was reduced by 23 ± 6% for 1 μM ionomycin and 34 ± 2% for 1 μM A23187 (mean ± SEM of four experiments), thus confirming that endocytosis of ^{125}I-tn is inhibited at high $[Ca^{2+}]_i$.

Effect of $[Ca^{2+}]_i$ Depletion and Elevation on Insulin Endocytosis

The endocytosis of ^{125}I-insulin by HL60 cells with resting $[Ca^{2+}]_i$ was found to occur slightly faster than that of ^{125}I-tn (Fig. 4). After a 15-min incubation at 37°C, 65% of total cell-bound insulin was internal. Cells with very low $[Ca^{2+}]_i$ showed almost identical kinetics of ^{125}I-insulin internalization as cells with resting $[Ca^{2+}]_i$, indicating again that endocytosis can occur in the virtual absence of cytosolic-free calcium. Although a slight decrease in the endocytosis of insulin was observed at high $[Ca^{2+}]_i$ (Figs. 4 and 5), this difference was not statistically significant ($P > 0.05$, $n = 4$).

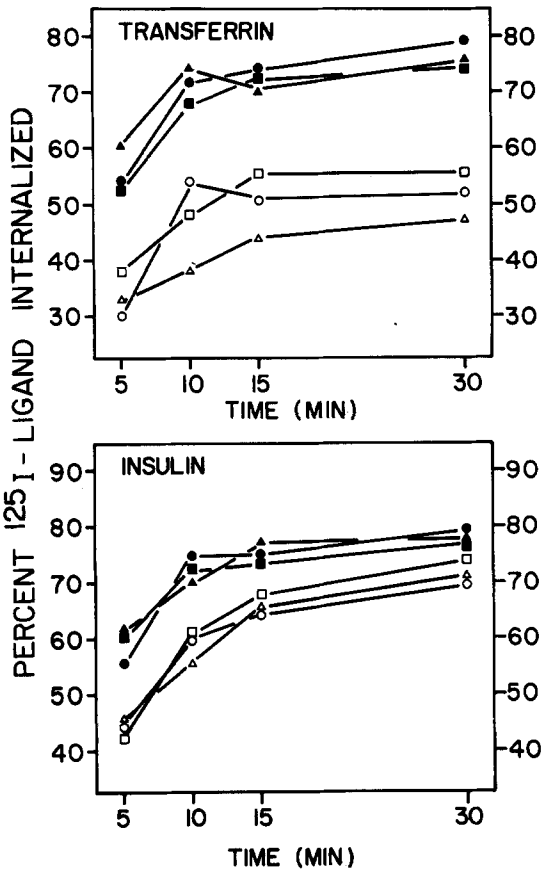

Figure 4. Effect of $[Ca^{2+}]_i$ and PMA on the kinetics of ^{125}I-tn and ^{125}I-insulin endocytosis. Cells in conditions of low, resting, and high $[Ca^{2+}]_i$ were obtained by quin2 loading in calcium-free medium, calcium medium, or by the addition of ionomycin as described in the Materials and Methods. They were then incubated with ^{125}I-tn or ^{125}I-insulin in the presence or absence of 50 nM PMA and the internalization of the ligands at the times shown determined by electron microscope autoradiography. The results given are the average of two separate experiments. □, resting $[Ca^{2+}]_i$; ○, low $[Ca^{2+}]_i$; △, high $[Ca^{2+}]_i$; ■, resting $[Ca^{2+}]_i$ + PMA; ●, low $[Ca^{2+}]_i$ + PMA; ▲, high $[Ca^{2+}]_i$ + PMA.

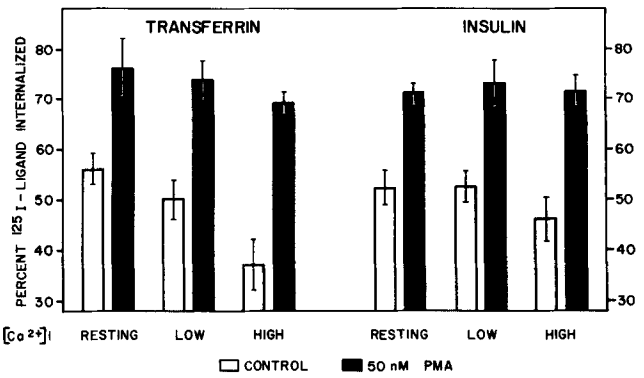

Figure 5. Stimulation of ^{125}I-tn and ^{125}I-insulin endocytosis by PMA at various $[Ca^{2+}]_i$. Low and high $[Ca^{2+}]_i$ cells were obtained, respectively, by quin2 loading in calcium-free medium or addition of ionomycin as described in the Materials and Methods. The cells were simultaneously incubated in the presence or absence of 50 nM PMA for 15 min at 37°C and with either ^{125}I-tn or ^{125}I-insulin. At the end of the incubation the cells were washed, processed for electron microscope autoradiography, and the percentage of internalized ligand determined as described in the Materials and Methods. Results shown are the mean ± SEM from four different experiments. Low $[Ca^{2+}]_i$ cells did not exhibit significantly lower internalization of transferrin and insulin than cells with resting $[Ca^{2+}]_i$ ($P > 0.05$). In conditions of high $[Ca^{2+}]_i$, transferrin endocytosis was significantly reduced ($P < 0.05$) but not that of insulin. PMA stimulated the internalization of both ligands in conditions of low, resting, and high $[Ca^{2+}]_i$ ($P < 0.05$ in all cases).

Phorbol Ester Stimulates Endocytosis of Transferrin

Previous studies have shown that the phorbol ester PMA stimulates the internalization, or "down-regulation," of unoccupied surface transferrin receptors (18, 23). In addition it has been shown using a biochemical technique that PMA increases the rate of transferrin endocytosis in immature erythroid cells (11). Since it is believed that the action of PMA is mediated via the enzyme protein kinase C (6) and that this enzyme is Ca^{2+} dependent (17), we examined the PMA stimulation of transferrin receptor internalization in conditions of low and high $[Ca^{2+}]_i$. As reported previously (18, 23), preincubation with PMA was found to reduce the surface binding of ^{125}I-tn by 40–50% (Figs. 2 and 6). This "down-regulation" of transferrin receptors was also induced by PMA in cells with low $[Ca^{2+}]_i$, suggesting it is a Ca^{2+}-independent process. Interestingly, and in agreement with a recent study by May et al. (18), cells with high $[Ca^{2+}]_i$ actually showed more down-regulation of surface transferrin receptors than cells with resting $[Ca^{2+}]_i$. As suggested by these authors, the high $[Ca^{2+}]_i$ could cause a redistribution of protein kinase C from the cell cytoplasm to the plasma membrane, thereby increasing the observed response to phorbol esters. The phorbol analogue, 4β-phorbol, had no effect on the surface binding of transferrin (Fig. 2).

Simultaneous incubation of HL60 cells at resting $[Ca^{2+}]_i$ with PMA stimulated the initial rate of endocytosis of ^{125}I-tn and increased the proportion of internalized ligand at steady state (Figs. 4 and 5). The proportion of total cell-bound transferrin, which was internal after 15-min incubation at 37°C, increased by an average of 20% in the presence of 50 nM PMA. PMA was also found to stimulate transferrin endocytosis in cells with either low or high $[Ca^{2+}]_i$ by about the same amount (Figs. 4 and 5), suggesting the effect was Ca^{2+}-independent.

Phorbol Ester Stimulates Insulin Internalization

In contrast to transferrin, preincubation with PMA had no effect on the surface binding of insulin to HL60 cells (Fig. 6), suggesting there was no down-regulation of unoccupied insulin receptors. However, simultaneous incubation with the phorbol ester at 37°C stimulated the internalization of ^{125}I-insulin to the same extent as that observed for transferrin (Figs. 4 and 5). As for ^{125}I-tn endocytosis, the PMA stimulation of insulin endocytosis was apparently independent of the $[Ca^{2+}]_i$.

Discussion

The regulation of receptor internalization and recycling remains a fundamental question in receptor-mediated endocytosis. At present, little is known about the biochemical mechanisms that modulate receptor movement to and from the cell surface. One of the possible candidates for such regulation

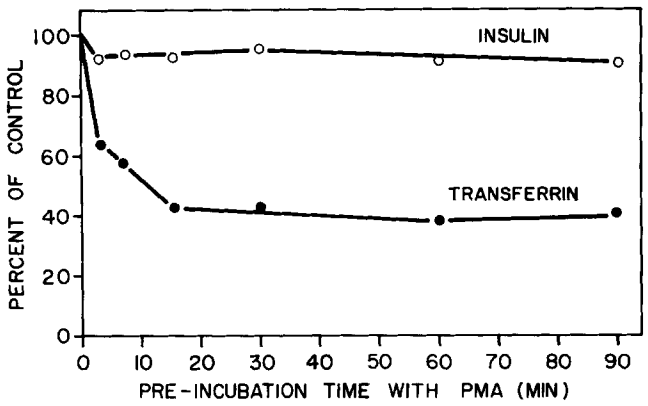

Figure 6. Effect of preincubation with PMA on the surface binding of ^{125}I-tn and ^{125}I-insulin. Cells were preincubated with 50 nM PMA at 37°C for the times shown. They were then washed twice with ice-cold PBS and the surface binding of ^{125}I-tn and ^{125}I-insulin determined by incubation at 4°C as described in the Materials and Methods. Results shown are the average values from duplicate incubations.

is the free cytosolic Ca^{2+} concentration. Receptor turnover involves processes such as selective membrane fusion and cytoskeletal rearrangements, events that are widely believed to be under the control of Ca^{2+}-dependent proteins. However several lines of evidence presented here indicate that, contrary to exocytosis, $[Ca^{2+}]_i$ plays no relevant role "per se" in the internalization of occupied transferrin and insulin receptors: (*a*) the rate and extent of transferrin- and insulin-receptor endocytosis, measured morphologically, is unaffected by loading the cells with micromolar concentrations of quin2 which are expected to blunt any localized gradients of Ca^{2+} within the cell; (*b*) reduction of $[Ca^{2+}]_i$ to very low values (10–15 nM), i.e., 5–10 times below normal resting levels, had no significant effect on the endocytosis of transferrin or insulin, conditions under which intracellular mobilizable Ca^{2+} stores are also depleted (7) and no known Ca^{2+}-regulated process is known to be operative.

Actually if Ca^{2+} has any role in receptor internalization it appears to be an inhibitory one, since elevation of $[Ca^{2+}]_i$ to micromolar concentrations with Ca^{2+} ionophores caused a small but reproducible inhibition of transferrin endocytosis (Figs. 4 and 5). Previous reports of reduced internalization of EGF (19, 22) and insulin-like growth factor (25) in the presence of A23187, as well as the inhibition of ^{59}Fe uptake at high $[Ca^{2+}]_i$, also support the conclusion that high $[Ca^{2+}]_i$ can inhibit the internalization of occupied surface receptors. We cannot rule out the possibility, however, that part of the inhibition of transferrin endocytosis was an apparent inhibition due to the increased surface binding at high $[Ca^{2+}]_i$ (Fig. 2 in this study and also reference 24). In absolute terms the amount of ligand internalized could be the same in the resting and high $[Ca^{2+}]_i$ conditions, however the percentage of internalized ligand would appear decreased in the latter because of the increased surface binding.

If a role for $[Ca^{2+}]_i$ "per se" in endocytosis seems to be excluded by the present data, a major role for protein kinase C in receptor internalization is on the other hand strongly supported by our experiments. Protein kinase C is an ubiquitous cytoplasmic enzyme that requires negatively charged phospholipids for activation. In vitro, protein kinase C is activated by high, unphysiological concentrations of Ca^{2+}, or by low concentrations of Ca^{2+} in the presence of either diacylglycerol or of active tumor promoters (6, 17), the most widely used being the phorbol diesters. A number of studies suggest that phorbol ester stimulation of protein kinase C can induce either internalization of surface receptors (2, 11, 18, 23, 29) or a decrease in receptor affinity (8, 10, 28, 32, 33). In the present experiments using quantitative electron microscope autoradiography, we have confirmed that transferrin endocytosis is stimulated by the active tumor promoter PMA (11). We have also shown that PMA stimulates the internalization of a very different ligand, insulin, to a similar degree as that observed for transferrin. This finding raises the possibility that activation of protein kinase C provides a general mechanism for regulating the internalization of occupied surface receptors. With both the occupied transferrin and insulin receptors, the PMA effect appeared to be independent of the $[Ca^{2+}]_i$ in that it was the same in resting, low, and high $[Ca^{2+}]_i$ states.

The reason why preincubation with PMA rapidly down-regulates unoccupied surface transferrin receptors but has no effect on the unoccupied insulin receptors is unclear. One possible explanation is that PMA, via its activation of protein kinase C, stimulates coated pits to internalize. Since unoccupied transferrin receptors have been shown to be concentrated in coated pits (12, 13, 38, 39) while unoccupied insulin receptors are mostly associated with microvilli (4, 5), this could account for the differential response to PMA. This model also explains the PMA stimulation of occupied insulin receptor internalization, since after insulin binding (4, 5) these receptors concentrate into coated pits. Further evidence supporting this model of PMA action is provided by the low density lipoprotein receptor which is concentrated in coated pits regardless of its state of occupancy (1, 9) and is similarly down-regulated by PMA in U-937 cells (29).

The precise calcium dependence of protein kinase C is unclear: Nishizuka and co-workers claim that in vitro, phorbol esters and diacylglycerol simply decrease the Ca^{2+} sensitivity of the enzyme, with Ca^{2+} remaining the ultimate trigger of protein kinase C (16). Under similar conditions however, Niedel et al. (26) and Di Virgilio et al. (7) showed that PMA can almost maximally stimulate protein kinase C–dependent phosphorylation at very low Ca^{2+} concentrations (i.e., $<10^{-8}$ M). The present data also agree with the interpretation that phorbol esters can stimulate cell functions independently of $[Ca^{2+}]_i$. In fact, the endocytosis of occupied transferrin and insulin receptors, and of unoccupied transferrin receptors, is stimulated by PMA to approximately the same extent regardless of whether $[Ca^{2+}]_i$ is below, at, or above the resting level. The insensitivity of the PMA effects to a 10-fold reduction of $[Ca^{2+}]_i$ below resting levels suggest that if, as largely accepted, PMA elicits its stimulation via protein kinase C–dependent phosphorylation(s), then this enzyme can be fully activated in intact cells even in the virtual absence of Ca^{2+}. More importantly, the steps subsequent to protein kinase C activation are also perfectly functional even in very low $[Ca^{2+}]_i$.

The major findings of this study can thus be summarized as follows: (*a*) the receptor-mediated endocytosis of two different types of ligands, transferrin and insulin, can proceed normally at very low $[Ca^{2+}]_i$; (*b*) the phorbol ester PMA stimulates the endocytosis of occupied insulin and

transferrin receptors; (*c*) this stimulatory effect of PMA on transferrin and insulin endocytosis, presumably mediated by activation of protein kinase C, is largely independent of the $[Ca^{2+}]_i$ in our intact cell system; (*d*) the PMA stimulation of endocytosis of unoccupied receptors is selective since unoccupied insulin receptors are not affected by the phorbol ester, whereas transferrin receptors are rapidly down-regulated. These data are therefore consistent with the notion that protein kinase C may be a major biochemical regulator of receptor-mediated endocytosis but are not consistent with a similar role for $[Ca^{2+}]_i$.

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