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 ([Ca2+]i) and of protein kinase C on the internalization of transferrin and insulin in the human promyelocytic cell line HL60 was investigated. [Ca2+]i was selectively monitored and manipulated by the use of the fluorescent Ca2+ indicator and buffer quin2, while receptor-ligand internalization was studied directly by quantitative electron microscope autoradiography. Decreasing the [Ca2+]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 [Ca2+]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 [Ca2+]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 [Ca2+]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.

U'PON 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 ([Ca2+]cyto) 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 [Ca2+]cyto, it has been suggested that localized Ca2+ gradients are involved in generating the signals for phagocytosis (21, 40) and that Ca2+-regulated processes could be involved in endocytosis (11, 30). Furthermore, increasing intracellular [Ca2+]cyto 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 Ca2+ indicator and chelator quin2 (34–36) has permitted the modulation and measurement of [Ca2+]cyto in small mammalian cells. One of the major biological applications of quin2 has been in the study of the role of [Ca2+]cyto 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 [Ca2+]cyto and protein kinase C in receptor turnover by using quin2 and Ca2+ ionophores (ionomycin, A23187) to respectively decrease and increase the [Ca2+]cyto, 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 [Ca2+]cyto, while activation of protein kinase C can selectively modulate receptor internalization.

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 FeCl3 per mole of transferrin. 100 µg of ferrotransferrin

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was radioiodinated with 1 mCi of 125I using Enzymobeads (Bio-Rad Laboratories, Richmond CA) according to the manufacturer's instructions. Radioiodinated transferrin (125I-tfn) was separated from free 125I using Sephadex G-25 gel chromatography. Human apotransferrin was also labeled with 59Fe as previously described (15). Radioiodinated A14 pork insulin (gift from Dr. Liu of Hoffman-La-Roche, Nutley, NJ) and A23187 (Sigma Chemical Co.) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and a performance liquid chromatography before lyophilization for shipment. The specific activities of the radiolabeled ligands were 4-6 Ci/µg for 125I-tfn and 250-350 Ci/µg for 59Fe-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 [Ca2+].

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 × 10⁶ cells/ml in medium containing 138 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.1 mM CaCl2, 100 µM EGTA, 1 mM NaHPO4, 5 mM NaHCO3, 3.5 mM glucose, and 20 mM Hepes, pH 7.4. This medium is referred to as calcium medium (Ca2+), = 10⁻⁷ M. In indicated experiments, calcium-free medium (Ca2+), = 10⁻⁸ 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 × 10⁷ 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. Intra-cellular quin2 concentrations were determined by comparing the Ca2+-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 [Ca2+]i were obtained by loading with quin2 in calcium medium as described above. Cells with low [Ca2+]i, were obtained by loading with quin2 in calcium-free medium, while high [Ca2+]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 in calcium medium, and incubated with radiolabeled 125I-tfn (5-10 µg/ml) or 125I-insulin (5-25 ng/ml) for up to 30 min at 37°C in the presence of 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 unlabelled ligand and comparing the radioactive uptake to cells incubated with the radiolabeled ligand. Radioactivity associated with cells in the presence of excess unlabelled ligand was considered to be due to nonspecific binding and accounted for no more than 6% of the 125I-tfn binding and 3% of the 125I-insulin binding.

Electron Microscope Autoradiography

At the end of the incubations with 125I-tfn or 125I-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.

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Figure 1. Depletion and elevation of [Ca2+]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 [Ca2+]i determined by measurement of the quin2 fluorescence. The [Ca2+]i calibration of the quin2 fluorescence is shown on the left side. Where indicated, 2 mM CaCl2 and 1 µM ionomycin were added, giving rise to resting [Ca2+]i, (115 nM) and high [Ca2+]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 approaches a line source of irradiation (31). To analyze a large number of grains, we simulated this line source model. Autoradiographic grains representing the 125I-tfn or 125I-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 125I-tfn or 125I-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 125I-tfn

Figure 2. The effect of [Ca2+]i and phorbol esters on the surface binding of 125I-tfn to HL60 cells. Cells in conditions of low, resting, and high [Ca2+]i, 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 125I-tfn for 30 min at 4°C. Also examined was the phorbol analogue 4β-phorbol. Results shown are the surface binding of 125I-tfn 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\textsuperscript{2+}]\textsubscript{i} incubated with \textsuperscript{125}I-tn for 15 min at 37°C, and (b) of a cell with resting [Ca\textsuperscript{2+}]\textsubscript{i} incubated with \textsuperscript{125}I-insulin for 15 min at 37°C.

Results

Depletion and Elevation of [Ca\textsuperscript{2+}]\textsubscript{i} in HL60 Cells

By loading HL60 with quin2 in calcium-free medium, the [Ca\textsuperscript{2+}]\textsubscript{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\textsubscript{2} to the medium the [Ca\textsuperscript{2+}]\textsubscript{i} returns to resting levels within ~5 min due to the influx of Ca\textsuperscript{2+} into the cell. Addition of 1 \mu M ionomycin to cells at resting [Ca\textsuperscript{2+}]\textsubscript{i} in calcium-containing medium results in a rapid and sustained 15–20-fold increase in the [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 1). Thus, by using the Ca\textsuperscript{2+} chelator quin2 and the Ca\textsuperscript{2+} ionophore ionomycin, the [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i}, Depletion and Elevation on Transferrin and Insulin Binding

Preliminary experiments showed that the quin2 loading procedure, in either calcium medium (resting [Ca\textsuperscript{2+}]\textsubscript{i}) or calcium-free medium (low [Ca\textsuperscript{2+}]\textsubscript{i}), caused no significant changes in the surface binding of \textsuperscript{125}I-tn (Fig. 2). However, by elevating the [Ca\textsuperscript{2+}]\textsubscript{i}, with either ionomycin or A23187, the surface binding of \textsuperscript{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 \textsuperscript{125}I-insulin to cells in conditions of low or high [Ca\textsuperscript{2+}]\textsubscript{i} was not significantly different to that of unloaded cells or quin2-loaded cells at resting [Ca\textsuperscript{2+}]\textsubscript{i} (results not shown).

Effect of [Ca\textsuperscript{2+}]\textsubscript{i}, Depletion and Elevation on Transferrin Endocytosis

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

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

Effect of [Ca\textsuperscript{2+}]\textsubscript{i}, Depletion and Elevation on Insulin Endocytosis

The endocytosis of \textsuperscript{125}I-insulin by HL60 cells with resting [Ca\textsuperscript{2+}]\textsubscript{i}, was found to occur slightly faster than that of \textsuperscript{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\textsuperscript{2+}]\textsubscript{i}, showed almost identical kinetics of \textsuperscript{125}I-insulin internalization as cells with resting [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i} (Figs. 4 and 5), this difference was not statistically significant (P > 0.05, n = 4).
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 (1). 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\(^{2+}\)]. As reported previously (18, 23), preincubation with PMA was found to reduce the surface binding of \(^{125}\)I-transferrin by 40-50% (Figs. 2 and 6). This "down-regulation" of transferrin receptors was also induced by PMA in cells with low [Ca\(^{2+}\)], 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\(^{2+}\)], actually showed more down-regulation of surface transferrin receptors than cells with resting [Ca\(^{2+}\)]. As suggested by these authors, the high [Ca\(^{2+}\)], 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, 4\(\beta\)-phorbol, had no effect on the surface binding of transferrin (Fig. 2).

Simultaneous incubation of HL60 cells at resting [Ca\(^{2+}\)], with PMA stimulated the initial rate of endocytosis of \(^{125}\)I-transferrin 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\(^{2+}\)], 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-transferrin endocytosis, the PMA stimulation of insulin endocytosis was apparently independent of the [Ca\(^{2+}\)].

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...
The free cytosolic $\mathrm{Ca}^{2+}$ concentration is the key regulator of receptor endocytosis. Receptor turnover involves processes such as selective membrane fusion and cytoskeletal rearrangements, events that are widely believed to be under the control of $\mathrm{Ca}^{2+}$-dependent proteins. However, several lines of evidence presented here indicate that, contrary to exocytosis, $\mathrm{Ca}^{2+}$ 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 $\mathrm{Ca}^{2+}$ within the cell; (b) reduction of $\mathrm{Ca}^{2+}$ levels 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 $\mathrm{Ca}^{2+}$ stores are also depleted (7) and no known $\mathrm{Ca}^{2+}$-regulated process is known to be operative.

Actually if $\mathrm{Ca}^{2+}$ has any role in receptor internalization it appears to be an inhibitory one, since elevation of $[\mathrm{Ca}^{2+}]$, to micromolar concentrations with $\mathrm{Ca}^{2+}$ 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 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 (II). 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 $[\mathrm{Ca}^{2+}]$, in that it was the same in resting, low, and high $[\mathrm{Ca}^{2+}]$, 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 $\mathrm{Ca}^{2+}$ sensitivity of the enzyme, with $\mathrm{Ca}^{2+}$ 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 $\mathrm{Ca}^{2+}$ concentrations (i.e., $<10^{-8} \text{ M}$). The present data also agree with the interpretation that phorbol esters can stimulate cell functions independently of $[\mathrm{Ca}^{2+}]$. 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 $[\mathrm{Ca}^{2+}]$, is below, at, or above the resting level. The insensitivity of the PMA effects to a 10-fold reduction of $[\mathrm{Ca}^{2+}]$ 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 $\mathrm{Ca}^{2+}$. More importantly, the steps subsequent to protein kinase C activation are also perfectly functional even in very low $[\mathrm{Ca}^{2+}]$.

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 $[\mathrm{Ca}^{2+}]$; (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\(^{2+}\)] 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\(^{2+}\)].

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