Phenylarsine Oxide-induced Increase in Alveolar Macrophage Surface Receptors: Evidence for Fusion of Internal Receptor Pools with the Cell Surface

JERRY KAPLAN, DIANE McVEY WARD, and H. STEVEN WILEY

Department of Pathology, University of Utah College of Medicine, Salt Lake City, Utah 84132

ABSTRACT Rabbit alveolar macrophages which were treated at 0°C with phenylarsine oxide and then incubated at 37°C for 10 min exhibited a two- to threefold increase in surface receptor activity for macroglobulin-protease complexes, diferric transferrin, and mannose-terminal glycoproteins. Analysis of the concentration-dependence of ligand binding indicated that changes in ligand-binding activity were due to changes in receptor number rather than alterations in ligand-receptor affinity. Surface receptor number could also be increased by treatment of cells with three other sulfhydryl reagents, N-ethylmaleimide, p-chloromercuribenzoate, and iodoacetic acid. The increase in receptor activity was maximal after 10 min and decreased over the next hour. This decrease in cell-associated receptor activity was due to the release of large membrane vesicles which demonstrated a uniform buoyant density by isopycnic sucrose gradient centrifugation. Treatment of cells with phenylarsine oxide did not decrease the cellular content of lactate dehydrogenase or β-galactosidase, indicating that cell integrity was maintained and lysosomal enzyme release did not occur. Our studies indicate that phenylarsine oxide treatment in the presence of extracellular Ca²⁺ results in the fusion of receptor-containing vesicles with the cell surface.

Studies indicate the existence of intracellular pools of plasma membrane receptors which can be recruited or exteriorized to the cell surface. These intracellular pools are thought to play a role in the recycling of membrane receptors. To date, intracellular pools of receptors have been demonstrated for asialoglycoproteins (1), mannose-terminal glycoproteins (2), insulin (3), transferrin (Tf) (4) and α-macroglobulin–protease complex (αM-P) (5), but not for low density lipoprotein receptors in fibroblasts (6) or for asialoglycoprotein receptors in cultured hepatoma cells (7). Examination of thin sections of cells by electron microscopy using anti-receptor antibodies (8) or homogenization or cells followed by subcellular fractionation (4) indicates that the intracellular reservoir of asialoglycoprotein or Tf receptors is associated with a unique low buoyant density organelle which may also contain recently internalized ligands. The biochemical properties of the intracellular compartment(s) containing the receptors are unclear.

During a study of the effect of sulphydryl reagents on endocytosis, we observed that treatment of alveolar macrophages with phenylarsine oxide (PAO), a vicinyl sulphydryl reagent, resulted in an increase in the binding of α-macroglobulin.125I-trypsin complex (αM·125I-T) to cell surface receptors. In this study we found that treatment of macrophages with PAO or other sulphydryl reagents at 0°C followed by incubation of cells at 37°C resulted in an increase in surface receptors for αM-P, Tf, and mannose-terminal glycoproteins. These data indicate that PAO increases surface receptor number by inducing the fusion of (an) intracellular receptor-containing compartment(s) with the cell surface.

MATERIALS AND METHODS

Cells: Rabbit alveolar macrophages were obtained by bronchial lavage and the cells prepared and cultured as previously described (9).

Abbreviations used in this paper: HBSS, Hanks' balanced salt solution; MAN-BSA, mannose–bovine serum albumin; MEM, minimal essential medium; αM·125I-T, α-macroglobulin·125I-trypsin complex; αM-P, α-macroglobulin–protease complex; NEM, N-ethylmaleimide; PAO, phenylarsine oxide; PCMB, p-chloromercuribenzoate; Tf(Fe)₃, diferric transferrin.
Ligands and Measurement of Binding Activity: α-Macroglobulin (αM) was isolated from either human or rabbit plasma and αM-125I-T was previously described (10). Mannone-bovine serum albumin (MAN-BSA) was a generous gift of Dr. C. Y. Lee. Rabbit Tf was prepared from rabbit plasma using the procedure of Sawatzki et al. (11). The Tf was iron loaded by incubation with ferric ammonium citrate and dialyzed against phosphate-buffered saline (PBS) to remove non-protein-bound iron. MAN-BSA and Tf were iodinated by a modification of the chloramine-T procedure of McConahey and Dixon (12). The binding of 125I-MAN-BSA or 125I-diferric transferrin, herein cited as 125I-Tf(Fe2), to macrophages was measured using the same procedure for assay of αM-125I-T binding to cells (9). The data presented for all experiments have been corrected for nonspecific binding, which was generally <10% of total binding. The figures and tables reflect representative experiments. However, all experiments were performed at least three times.

Preparation of Samples for Light and Electron Microscopy: For examination of cells by either light or electron microscopy, cells were plated on glass coverslips and subjected to the manipulations described in the figure legends. For examination by light microscopy, cells were fixed with 2% glutaraldehyde in cacodylate buffer and then examined by phase-contrast optics using a Zeiss microscope.

For electron microscopic examination, cells were placed into Karnovsky's fixative (pH 7.4) for 30 min, washed with PBS, and then incubated with 2.0% osmium tetroxide for 30 min. The coverslips were washed with saline and then dehydrated with a graded series of acetone, incubated in a mixture of 100% acetone and 2% osmium tetroxide for 30 min each, and then air dried. Thin sections were prepared, stained with uranylacetate, and examined by transmission electron microscopy using a Siemens electron microscope.

Additional Procedures: Protein determinations were performed as described by Lowry et al. (13) using BSA fraction V (Sigma Chemical Co., St. Louis, MO) as a standard. The activities of β-galactosidase, alkaline phosphodiesterase, and lactate dehydrogenase were measured as described by Kaplan (14), Edelson and Erbs (15), and Bergmeyer et al. (16), respectively. Homogenization of cells and measurement of ligand-binding activity to membrane vesicles obtained from homogenates were performed as previously described (9). Sucrose gradients were prepared as previously described (17). Sucrose density was measured at 22°C using a Bausch and Lomb refractometer. ATP determinations were performed as described by Addanki et al. (18).

Materials: N-ethylmaleimide (NEM) was obtained from Pierce Chemical Co. (Rockford, IL). Iodoacetic acid, p-chloromercuribenzoate (PCMB), luciferin-luciferase, and PAO were obtained from Sigma Chemical Co. PAO was dissolved in dimethylsulfoxide prior to use.

RESULTS

Treatment of Cells with Sulphydryl Reagents Increases Surface Receptor Number

Treatment of cells with 0.1 mM PAO at 0°C for 60 min results in a complete inhibition of receptor-mediated internalization of 125I-epidermal growth factor in human fibroblasts (19) and αM-125I-T in alveolar macrophages when cells are shifted to 37°C (Fig. 1). Continuous incubation of PAO-treated macrophages at 0°C did not result in any alteration in binding activity for either αM-P (125I-Tf(Fe2)), or MAN-BSA (Fig. 2). However, when PAO-treated cells were incubated at 37°C for 10 min and then shifted back to 0°C to measure ligand binding, there was a two- to threefold increase in binding activity for all three ligands (n = 19). Increases in αM-P receptor number could be observed (n = 9) within 2–3 min of exposure of PAO-treated cells to 37°C. Receptor number reached a maximum within 10–15 min of incubation at 37°C followed by a decline over the subsequent hour (Fig. 3). Unless otherwise noted, changes in receptor number were examined after incubation of treated cells at 37°C for 10–15 min.

Previously, we demonstrated that shifting the incubation temperature of macrophages (0°C → 37°C → 0°C) resulted in a one-time-only 40–60% reduction in surface receptor number (9). Exposure of cells to a temperature shift prior to treatment with PAO did not prevent the observed increase in receptor number although the maximum number measured was less than that of cells not exposed to the temperature shift (data not shown).

PAO-induced increases in αM-125I-T-binding activity were observed (n = 4) using concentrations as low as 1–2 × 10−3 M (Fig. 4A). Increased ligand-binding activity was not restricted to PAO treatment alone but could be observed in cells treated with other sulphydryl reagents. The data in Fig. 4 demonstrate that treatment of cells with NEM (Fig. 4B), iodoacetic acid, or PCMB (data not shown) had no effect on binding activity in cells maintained at 0°C, whereas incubation of treated cells at 37°C for 10 min resulted in increased binding activity. The increase in binding activity for all four sulphydryl reagents was concentration dependent. Among the agents tested, the largest effect was obtained with PAO and thus this compound was chosen for further study.

Increased ligand-binding activity could result either from a change in surface receptor number or an alteration in ligand-receptor affinity. To distinguish between these possibilities, we measured the binding of ligands to cells as a function of
ligand concentration. Treatment of cells with PAO at 0°C for 60 min followed by further incubation at 0°C did not affect the concentration dependency of binding of either $^{125}$I-MAN-BSA or $\alpha$M-$^{125}$I-T (Fig. 5). Brief incubation (10 min) at 37°C of untreated control cells did not increase binding activity. However, certain cells were treated with PAO at 0°C, incubated for 10 min at 37°C, and then returned to 0°C; these cells bound more ligand than control cells at each ligand concentration tested. In all instances ($n = 4$), the ligand concentration required for half-maximal saturation (3 nM for $\alpha$M-$^{125}$I-T and 2 nM for $^{125}$I-MAN-BSA) was unaffected. These results indicate that increased binding activity resulted from a change in receptor number. Increases in receptor number were also observed ($n = 3$) in membrane vesicles prepared from cellular homogenates obtained from PAO-treated cells exposed to 37°C, but not in vesicles of cellular homogenates from PAO-treated cells maintained at 0°C, control cells maintained at 0°C, or control cells exposed to 37°C for 10 min (Table I). Since the majority of ligands binding to vesicles can be ascribed to plasma membrane receptors (9), this result further suggests that treatment of cells with PAO at 0°C followed by a brief incubation at 37°C results in the appearance of new receptors on the cell surface and is not merely the result of changes in cell shape.

Evaluation of a number of morphologic and biochemical parameters indicated that brief incubations of PAO-treated cells at 37°C did not affect cellular integrity. Examination of PAO-treated cells by phase-contrast microscopy revealed a slight flattening or spreading after incubation at 37°C for 10 min. With further incubation at 37°C (30-60 min), the cells started to bleb and subsequently release large clear membrane vesicles into the media (data not shown). These “blebs” appeared indistinguishable from blebs released by other cell types after treatment with sulfhydryl reagents (20). A 10-min incubation of PAO-treated cells at 37°C did not affect trypan blue staining. Similar percentages of control and treated cells

---

**Figure 3** Kinetics of the PAO-induced increase in receptor number in macrophages incubated at 37°C. Cells were incubated in HBSS at 0°C for 60 min in the presence or absence of 0.1 mM PAO. The cells were washed, resuspended in MEM, and aliquots were incubated at 37°C. At specified times, samples were removed, centrifuged, cell pellets resuspended and incubated at 0°C for 60 min with $\alpha$M-$^{125}$I-T to determine specific binding. Binding of $\alpha$M-$^{125}$I-T to control cells incubating at 37°C (O) and PAO-treated cells incubated at 37°C ( ●).

**Figure 4** Concentration dependence of surface receptor increases by sulfhydryl reagents. Cells plated on tissue culture dishes were incubated in the specified concentrations of sulfhydryl reagents for 60 min at 0°C. Cells were then washed four times with cold HBSS, and either maintained at 0°C or 37°C for 10 min. The cells were then placed at 0°C, washed with cold HBSS, and binding was determined with $\alpha$M-$^{125}$I-T as a ligand. Control cells at 0°C (A), control cells at 37°C, 10 min (△), sulfhydryl-treated cells at 0°C (●), sulfhydryl-treated cells at 37°C, 10 min (○). Cells were treated with PAO (A) and NEM (B).

**Figure 5** Concentration dependence of $\alpha$M-$^{125}$I-T or $^{125}$I-MAN-BSA binding to control and PAO-treated cells. Cells were incubated in HBSS at 0°C for 60 min in the presence or absence of 0.1 mM PAO. The cells were then washed with HBSS and aliquots of control and PAO-treated cells were incubated at 37°C for 10 min. The cells were then placed at 0°C and incubated with different concentrations of $\alpha$M-$^{125}$I-T (A) for 60 min or $^{125}$I-MAN-BSA (B) to determine specific binding. Specific binding of the radiolabeled ligand to control cells (●) and PAO-treated cells (○) maintained at 0°C, or control cells (△) and PAO-treated cells (○) incubated at 37°C for 10 min.
were then washed twice with cold HBSS, and an aliquot of 0.1 M NaCl, pH 8.0.

Electron microscopic examination of PAO-treated cells incubated at 37°C for 10 min revealed no striking abnormalities in membrane integrity or any evidence of cell dissolution (data not shown). These morphological observations were supported by biochemical studies demonstrating that short incubations at 37°C did not result in losses of cytosolic, plasma membrane, or lysosomal contents (Fig. 6). Cellular ATP content was also unaffected in PAO-treated cells exposed to 37°C for 10 min. Control cells contained 3.1 ± 0.1 × 10⁻⁶ mol ATP/mg protein, a value close to that reported previously (21). PAO-treated cells incubated at 37°C for 10 min contained 2.9 ± 0.2 × 10⁻⁶ mol ATP/mg protein (n = 3). These observations indicate that a short incubation of PAO-treated cells at 37°C did not affect membrane integrity.

**Studies on the Mechanism of Increased Surface Receptor Number**

Experiments were performed to identify the mechanism(s) responsible for the increased receptor number resulting from exposure of PAO-treated cells to 37°C. The receptors assayed in this study are involved in ligand accumulation and have been demonstrated to recycle (21). Increases in surface receptor number could result from either a specific inhibition of internalization or an acceleration of exocytosis/recycling. We initially considered the possibility that PAO treatment affected the rate of receptor internalization but not the rate of receptor reappearance at the cell surface. Consequently the increased number of surface receptors would reflect depletion of an internal pool. Evidence suggesting this hypothesis arises from the observation that the concentrations of PAO that lead to increased receptor number are those that inhibit endocytic activity (data not shown). However, a number of experimental approaches indicate that increased receptor number did not result from inhibition of endocytosis.

Incubation of macrophages with NaCN or NaCN plus NaF results in a reduction of cellular ATP pools by 80% (22) and inhibition of internalization of surface-bound αM-¹²⁵I-T (17) (Fig. 7B). Cell surface receptor number is only slightly affected by reductions in cellular ATP content (Fig. 7A). However, cells treated with metabolic poisons do show (n = 3) an increased receptor number when exposed to PAO at 0°C and shifted to 37°C for 10 min (cf. Fig. 7C and D). Thus, inhibition of internalization, per se, does not lead to increased receptor number.

The inhibition of internalization of surface-bound αM-¹²⁵I-T by PAO is independent of extracellular calcium (data not shown). Cells that were treated with PAO at 0°C and incubated at 37°C for 10 min in the absence of calcium and magnesium were incapable of internalizing ligands when subsequently incubated in calcium-containing solutions. Measurement of

---

**TABLE 1. Demonstration of Increased Ligand-binding Activity on Membranes Prepared from Homogenates of PAO-treated Cells**

| Specific Activity | fmol/µg protein⁻¹ ± SD |
|------------------|------------------------|
| 0°C              | 37°C                   |
| Whole cells      |                        |
| Control          | Control                |
| PAD              |                         |
| Membrane vesicles|                        |
| Control          | Control                |
| PAD              |                         |

Cells were treated with or without 0.1 mM PAO for 60 min at 0°C. Cells were then washed twice with cold HBSS, and an aliquot of control and PAO cells was shifted to 37°C for 10 min. Samples were then washed once in cold 10 mM Tris-HCl, 0.15 M NaCl, pH 8.0, and aliquots were removed for whole cell binding measurements. The remaining cells were homogenized at setting 7 with two 30-s bursts using a Brinkmann Polytron. Membranes were centrifuged at 200 g for 10 min, supernatants were kept and centrifuged at 10,000 g for 20 min, and pellets were resuspended in 10 mM Tris-HCl, 0.15 M NaCl, pH 8.0.

Binding assays on whole cells and membrane vesicles were done at 0°C using αM-¹²⁵I-T as the ligand.

---

**FIGURE 6** Effect of PAO treatment on levels of macrophage enzymes and binding activity. Cells plated in tissue culture dishes were incubated in HBSS at 0°C for 60 min in the presence or absence of 0.1 mM PAO. Cells were then washed twice with cold HBSS and 2 ml of Hanks' MEM was added to each plate. One set of plates was maintained at 0°C and the other was incubated at 37°C for 10 min and then returned to 0°C. The media was removed for enzyme analyses. Binding studies were done on cells incubated at 0°C using αM-¹²⁵I-T. Cells were solubilized in 0.1% Triton X-100 and enzyme assays were performed on the cell extract and the media. Activity in control cells (white bars) and PAO cells (striped bars). Activity in media are the smaller bars to the right of respective larger bars except in the panel showing the binding of αM-¹²⁵I-T.
surface receptor number in such cells revealed only slight increases in receptor number (Table II). However, cells subjected to similar protocol (modified by the presence of calcium and magnesium during both the 0°C and 37°C incubations) demonstrated (n = 3) a two- to threefold increase in receptor number.

Incubation of cells with agents which increase the pH of internal acidic compartments results in a ligand-independent loss of surface receptors and a decrease in the rate of receptor recycling (2, 6, 9, 23, 24). Thus, if continual recycling of receptors is responsible for the increase in receptor activity, these agents should block the observed effects of PAO. As shown in Table III, incubation of macrophages with methylamine alone resulted in a 50% reduction in αM-P-binding activity in agreement with our previous findings (25). When cells pretreated with methylamine were subsequently treated with PAO, the cells still exhibited the increase in receptor number (n = 3). These results suggest that the PAO-induced increase in receptor number was not the result of continued recycling in the face of an inhibition of endocytosis.

Studies indicate that internalized ligands are directed to a nonlysosomal membrane-bound compartment prior to their appearance in lysosomes. Lamb et al. (4) and Geuze et al. (8) have demonstrated that this “intermediate vesicle” or endosome also contains receptors. Consequently, if cells are incubated at 37°C for short periods of time, the bulk of the internalized ligand should be in the intermediate vesicle compartment(s). If PAO induces fusion of this vesicle with the plasma membrane, previously internalized ligand should be released into the media and thus lost from the cell monolayer. To determine whether PAO induces the fusion of this compartment with the cell surface, the following experiment was performed. Macrophages were incubated with αM-125I-T at 37°C for 15 min. The cells were washed with cold EDTA-containing solutions to remove the surface-bound ligand (17), incubated at 0°C for 60 min in the presence or absence of 0.1 mM PAO, and then placed at 37°C for 10 min in the presence of calcium. The cell monolayers were then assayed for the loss of radioactivity. PAO treatment of cells that had been “pulsed” for 15 min with αM-125I-T resulted in the loss of 50% of cell-associated radioactivity when such cells were shifted to 37°C for 10 min (Fig. 8A). However, little cellular loss of radioactivity was observed in cells that had been pulsed for 15 min, incubated at 37°C in media free of ligand for 60 min (“chased”), and then treated with PAO (Fig. 8B). This latter observation indicates again that PAO treatment does not result in release of lysosomal contents.

Dunn et al. (26) demonstrated that hepatocytes incubated at 18°C could internalize asialoglycoproteins, but that the ligand was not degraded in lysosomes. We have confirmed this observation by demonstrating that when macrophages whose receptors are occupied by αM-125I-T or 125I-MAN-BSA are incubated at 18°C, surface-bound ligands are internalized with a τ0 of 10 min, but no acid-soluble radioactivity is released for at least 3 h. When cells containing internalized radiolabeled ligands are shifted to 18°C, the generation of acid-soluble radioactivity continues, albeit at a slower (~20% control) rate. We have demonstrated that transfer of internalized αM-125I-T from endosome-enriched fractions to lysosomes is prohibited or delayed in cells incubated at 18°C by using Percoll gradients to separate subcellular organelles (Ajioka, R., D. M. Ward, S. Buys, and J. Kaplan, manuscript in preparation). Thus, if vesicle lysosome fusion is not totally inhibited it clearly is delayed at the lower temperature. As demonstrated in Fig. 8C, PAO treatment resulted in the loss

**Figure 7** The effect of PAO on surface receptor number in cells treated with metabolic inhibitors. (A) Cells plated on tissue culture dishes were incubated in MEM at 37°C, 60 min in the presence of 10^-3 M NaCN (dark bar) or 10^-3 M NaCN + 10^-3 M NaF (striped bar), or in the absence of these metabolic inhibitors (white bar). Cells were then placed at 0°C and washed extensively with HBSS. Binding activity was determined on cells incubated at 0°C using αM-125I-T. (B) Cells were treated as in A. After incubation with αM-125I-T at 0°C, cells were washed four times with cold HBSS, 1 ml MEM was added back to plates, and plates were then incubated at 37°C for 10 min. The media was removed and plates were washed twice at 0°C with 1 ml Ca²⁺, Mg²⁺-free HBSS containing 5 mM EDTA to remove the surface-bound ligand. Specific activities shown represent EDTA-resistant activity (i.e., internalized ligand). (C and DI Cells treated with metabolic inhibitors as in B were placed at 0°C and washed four times with cold HBSS. Cells were then maintained at 0°C in the absence or presence of 0.1 mM PAO (D). The cells were then washed extensively, 1 ml MEM added, and the plates were incubated at 37°C for 10 min. The cells were washed at 0°C and binding studies performed using αM-125I-T.

**Table II.** The Effects of Extracellular Ca²⁺ and Mg²⁺ on the PAO-induced Appearance of Receptor Activity

| Sample | Specific activity (fmol·µg protein⁻¹ ± SD) |
|--------|----------------------------------------|
|        | 0°C       | 37°C     | 0°C       | 37°C     |
|        | +Ca²⁺     | -Ca²⁺    | +Ca²⁺     | -Ca²⁺    |
| **Control** | 0.208 ± 0.010 | 0.172 ± 0.010 | 0.198 ± 0.005 | 0.182 ± 0.006 |
| **PAO**   | 0.177 ± 0.030 | 0.577 ± 0.027 | 0.154 ± 0.009 | 0.204 ± 0.013 |

Cells were plated on tissue culture dishes and incubated at 37°C in MEM for 60 min. The cells were then placed at 0°C and washed with and incubated in either HBSS or Ca²⁺, Mg²⁺-free HBSS. The cells were then incubated in the presence or absence of PAO in the above solutions. After 60 min, the cells were then washed with either HBSS or Ca²⁺, Mg²⁺-free HBSS, and incubated at 37°C for 10 min in those solutions. The cells were placed at 0°C, washed twice with cold HBSS, and the specific binding of αM-125I-T was then determined.

KAPLAN ET AL. Increase in Surface Receptors 125
TABLE III. Effect of Incubation of Cells with Methylamine on the PAO-induced Increase in aM-125I-T Receptor Activity

| Treatment      | Specific activity (fmol/μg protein) ± SD |
|----------------|-----------------------------------------|
|                | 0°C | 37°C, 10 min | 37°C, 30 min |
| Control        | 0.103 ± 0.003 | 0.083 ± 0.010 | 0.071 ± 0.004 |
| Methylamine    | 0.093 ± 0.000 | 0.084 ± 0.005 | 0.057 ± 0.011 |
| PAO            | 0.092 ± 0.004 | 0.263 ± 0.011 | 0.183 ± 0.000 |
| Methylamine/PAO| 0.091 ± 0.007 | 0.254 ± 0.001 | 0.190 ± 0.002 |

Cells were incubated at 37°C for 30 min in the presence or absence of 5.0 mM methylamine. The cells were then washed twice with cold HBSS and incubated in the presence or absence of 0.1 mM PAO for 60 min at 0°C. The cells were then washed twice with cold HBSS, resuspended in Hanks' MEM, and aliquots were either maintained at 0°C and incubated to 37°C for 10 min, or incubated at 37°C for 30 min. All samples were then washed twice with cold HBSS and specific binding was determined at 0°C for 60 min using aM-125I-T as the ligand.

FIGURE 8 Effect of PAO on the release of internalized aM-125I-T. Cells plated on tissue culture dishes were incubated at 37°C for 15 min with 1 x 10⁻⁸ aM-125I-T. After this 15 min pulse, cells were placed at 0°C, washed with cold Ca²⁺, Mg²⁺-free HBSS containing 5.0 mM EDTA to remove surface-bound ligand, and then incubated at 0°C in HBSS in the presence or absence of 0.1 mM PAO for 60 min. Cells were then washed with cold HBSS, placed in Hanks' MEM and incubated for 10 min either at 37°C or 0°C. Supernatants were removed and the cells were washed with Ca²⁺, Mg²⁺-free HBSS with 5.0 mM EDTA. The amount of radioactivity in the supernatant, EDTA wash, and in the monolayer cell was determined. The latter EDTA wash was done to remove any previously internalized radioactivity that had now been exteriorized but remained bound to surface receptors. For these experiments, radioactivity present in the washes has been normalized to the amount of monolayer protein. Stripped bars represent combined radioactivity. White bars represent combined radioactivity in the media and in the EDTA wash. However, insignificant amounts of radioactivity were detected in the EDTA wash.

The data in Fig. 3 demonstrate that after incubation of PAO-treated cells at 37°C, surface receptor number reached a maximum after 5–10 min and declined over the subsequent hour. The decline in cell surface receptors was correlated with the appearance of membrane-associated receptor activity in the media. The extracellular receptor activity could be determined simply by measuring binding activity in the pellet from media centrifuged at 10,000 g (20 min). The loss of cellular activity was mirrored by the increase in extracellular receptor activity. After incubation of PAO-treated cells for 5 min at 37°C, no aM-P receptor activity could be detected in the media, whereas appreciable amounts of receptor activity could be detected after a 60-min incubation (Table IV). The correlation was essentially quantitative when aM-P receptor activity was assayed. However, loss of surface Tf receptor activity was not accompanied by an equivalent increase in Tf-binding activity in the media. Among the possible explanations for this observation are differences in protease sensitivity (the Tf receptor is trypsin and pronase susceptible, and the aM-P receptor is protease resistant [Kaplan, J., unpublished observations]) or changes in receptor affinity. Nevertheless, declines in cell-associated Tf receptor activity were always accompanied by increases in extracellular receptor activity.

In addition to receptor activity, we observed the appearance in the medium of the plasma membrane ectoenzyme alkaline phosphodiesterase. The kinetics of appearance of extracellular activity matched the kinetics of appearance of extracellular receptor activity. Similar concentrations of PAO, which affect receptor distribution, also result in the appearance of extracellular enzyme activity (Fig. 9). When media from PAO-

TABLE IV. Relationship between the PAO-induced Loss of Receptor Activity and Intracellular Receptor Activity

| Specific Activity | fmol/μg protein ± SD |
|------------------|----------------------|
| aM-P             |                      |
| Control cells    | 0.022 ± 0.002       |
| PAO cells        | 0.010 ± 0.001       |
| Control media    | N.D.                |
| PAO media        | N.D.                |
| Tf(Fe₂)          |                      |
| Control cells    | 0.131 ± 0.010       |
| PAO cells        | 0.154 ± 0.002       |
| Control media    | N.D.                |
| PAO media        | 0.002 ± 0.002       |

* N.D. = not detectable (<0.002 fmol·μg protein⁻¹).

Alveolar macrophages were incubated in Hanks' MEM for 45 min at 37°C. The cells were centrifuged and the pellet resuspended in HBSS. An aliquot of cells was treated with PAO (0.1 mM, 0°C, 60 min) and the other aliquot was incubated at 0°C for 60 min in HBSS. Cells were then washed twice with cold HBSS, resuspended in Hanks' MEM, and incubated at 0°C, 10 min; 37°C, 10 min; 37°C, 60 min. Cells were then centrifuged at 200 g and aliquots of the supernatant and cells were incubated with aM-125I-T or 125I-Tf(Fe₂) to determine receptor binding. Receptor-binding activity in the supernatant was determined using a protocol similar to that used to assay cellular receptors with the exception that samples were centrifuged at 8,000 g for 1 min to separate the membrane-bound ligand from the free ligand. The data has been normalized to cellular protein. What is presented is a representative experiment in which 125I-Tf(Fe₂) and aM-125I-T binding activity were measured on the same population of cells.

of 60% of aM-125I-T accumulated in cells incubated for 60 min at 18°C. These results are consistent with the view that PAO treatment effects the fusion of receptor-containing vesicles with cell surfaces, and that the vesicles are at least a transient repository for the internalized ligand.
treated as in Fig. 4 using PAO as the sulfhydryl reagent except that to determine the amount of activity released into the media. Activity after incubation at 0°C or 37°C for 60 min, supematants were removed and cells solubilized in 0.1% Triton X-100. Alkaline phosphodiesterase activity was assayed in supernatants and cell extracts to determine the amount of activity released into the media. Activity in control media, 0°C ( ); control media, 37°C ( ); media from PAO-treated cells incubated at 0°C ( ); and from PAO-treated cells at 37°C ( ). The data is expressed as percent activity relative to the amount of activity in control of PAO-treated cells maintained at 0°C. In cells maintained at 0°C, PAO treatment did not affect alkaline phosphodiesterase activity.

**DISCUSSION**

PAO and other sulfhydryl reagents covalently modify cellular sulfhydryl groups, and although their exact site of action is unknown, one consequence of sulfhydryl modification is the irreversible inhibition of endocytosis. The ability of a sulfhydryl reagent to inhibit endocytosis has allowed for an analysis of surface receptor ligand interactions of the metabolic events that usually accompany ligand binding (i.e., ligand internalization) (cf. reference 19). This study demonstrates another effect of sulfhydryl reagents: the specific fusion of (an) intracellular receptor-containing compartment(s) with the cell surface.

These results demonstrate that treatment of alveolar macrophages with PAO or other sulfhydryl modifiers at 0°C followed by a brief incubation at 37°C causes marked increases in the number of three different receptors at the cell surface. Binding activity for Tf(Fe)₂, αM-Pr, and MAN-BSA increased two- to threefold within 10 min of incubation at 37°C followed by a decline in receptor number over the next hour. The initial increase in ligand-binding activity was due to an increase in the number of cell surface receptors as opposed to an alteration in ligand-receptor affinity.

Two hypotheses can account for the PAO-induced increase in receptor number. The first is that PAO inhibits endocytosis but does not prevent the return of internalized receptors to the cell surface. The following observations, however, argue against this hypothesis: (a) inhibition of internalization by exposure of cells to energy poisons does not lead to increases in surface receptor number, and (b) though the inhibitory effect of PAO on receptor internalization is calcium and magnesium independent, the increase in receptor number requires that cells be treated with the drug and incubated at 37°C in the presence of extracellular calcium and magnesium. The second hypothesis is that PAO induces the fusion of an intracellular receptor-containing vesicle with the cell surface. This hypothesis is supported by the following observations: (a) PAO treatment does not result in the release of a significant fraction of cellular lactate dehydrogenase or β-galactosidase; (b) PAO treatment induced the release of ligand which was internalized during a pulse but no release was observed if the "pulse" was followed by a chase; and (c) PAO could induce an increase in receptor number in cells pretreated with methylamine, an agent demonstrated to inhibit or alter receptor recycling. The appearance of receptors on methylamine-treated cells exposed to PAO could conceivably be due to a PAO-induced "reactivation" of vesicle acidification. We find this hypothesis unlikely in view of studies demonstrating that sulfhydryl modifiers inhibit vesicle acidification in vitro (27).

Rather, our data suggest that PAO treatment and exposure of PAO-treated cells to 37°C results in the fusion of (an) intracellular compartment(s) with the cell surface. The data further indicate a correlation between the compartment that contains intracellular receptors and a nonlysosomal compartment that contains internalized ligands. These observations support studies in HeLa cells (4) and isolated hepatocytes (8) which demonstrate that intracellular receptors and newly internalized ligand are present in the same compartment.

Extended incubation of PAO-treated cells at 37°C resulted in the release of membrane vesicles and a concomitant decrease in surface receptor number. Scott et al. (20) observed that treatment of cells with a variety of sulfhydryl reagents resulted in the release of membrane vesicles. Indeed, examination of the literature reveals numerous instances in which treatment of cells with sulfhydryl modifiers resulted in membrane blebbing (28, 29). Scott et al. (30) observed that the
membrane components present in the released vesicles were not selective but represented a random distribution of membrane polypeptides. They further demonstrated that release of membrane vesicles was calcium dependent. We observed that the increase in surface receptor number as well as the release of membrane components was calcium and magnesium dependent whereas the inhibition of endocytic activity by PAO was calcium and magnesium independent.

The observation that the PAO-induced increase in receptor number was calcium dependent may indicate the mechanism responsible for the phenomenon. The literature is replete with studies demonstrating that under the appropriate conditions sulfhydryl modifiers can affect membrane-associated processes. For example, Brachet et al. (31) demonstrated organomercurials in the presence of calcium-induced meiosis in Xenopus laevis oocytes. Kono et al. (32) reported that insulin induces the fusion of glucose transporter that contain intracellular vesicles with adipocyte cell surfaces, and that similar translocations of glucose transporters occurred in cells treated with PCMB. Bindoli et al. (33) observed that treatment of isolated muscle sarcoplasmic reticulum with a sulfhydryl reagent resulted in a selective loss of stored calcium and concluded that sulfhydryl reagents could affect or open calcium channels. These observations lead us to suggest the hypothesis that perhaps the sulfhydryl reagents cause an increase in intracellular calcium and that intracellular calcium induces the fusion of (an) intracellular vesicle(s) with the cell surface. Support for this hypothesis can be found in the observation that the calcium ionophore A 23187 in the presence of extracellular calcium can effect meiotic maturation in Xenopus oocytes (34) as well as induce the fusion of intracellular compartments with the cell surface (35). Recently, we determined that macrophages demonstrate increased cell surface receptors for αM·P and Tf upon exposure to A 23187 (36). This effect was reversible and dependent on extracellular calcium. PAO could be the most effective sulfhydryl reagent because it may permeabilize cells to calcium without perturbing the components necessary for fusion. Alternatively, the cell membrane may simply be more permeable to PAO than to other sulfhydryl reagents (37).

Though the mechanism behind the fusion of intracellular compartments with the cell surface is unclear, these data do demonstrate the existence of internal pools of receptors. In each instance the cells had not been previously exposed to ligands, which suggests that the existence of intracellular pools is native and not ligand induced. These internal pools may play a role in receptor recycling. In a previous study, we demonstrated that αM·P receptors which are in an internal compartment could be recruited to the cell surface (5). This was accomplished by using photoaffinity agents to cross-link αM·P complexes to surface receptors and then measuring the rate of appearance of unoccupied receptors on cell surfaces. Other studies indicate that receptors in an internal compartment may be recruited to the cell surface (1). At present we do not have a precise measurement of the size of the internal receptor pool although at a minimum it must contain at least twice the complement of surface receptors (cf. Fig. 3).

The observation that treatment of cells with pharmacological agents induces the fusion of receptor-containing intracellular compartments with the cell surface leads to the speculation that this type of fusion event may also occur under normal physiological circumstances. Recent studies indicate that endosome/membrane fusion in fibroblasts is an early response to epidermal growth factor (38) and to phorbol esters in macrophages (36). Petty et al. (39) hypothesized that an internal reservoir of membrane must exist to replace the plasma membrane which is internalized as a result of phagocytosis to IgG-coated liposomes by murine macrophages. We suggest that the internal compartment of receptors which PAO causes to fuse with the cell surface may be that internal reservoir. Experiments are in progress testing this hypothesis.

The authors would like to express their appreciation to Jeanne Novak for supplying rabbit transferrin; Janis Larson for her electron microscopy work; and Ms. E. Hart, J. Lawton, and M. L. Warren for preparing this manuscript.

This study was supported by a grant from the National Institutes of Health (HL2592203) and in part by a grant from R. J. Reynolds Industries. J. Kaplan is a recipient of a Research Career Development Award (HL 0084103).

Received for publication 15 November 1984, and in revised form 27 March 1985.

REFERENCES

1. Bridges, K., J. Harford, G. Ashwell, and R. D. Klauon. 1982. Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes. Proc. Natl. Acad. Sci. USA. 79:350-354.
2. Tietze, C., P. Schlesinger, and P. Stahl. 1982. Mannose-specific endocytosis receptor of alveolar macrophages: demonstration of two functionally distinct intracellular pools of receptors and their role in receptor recycling. J. Cell Biol. 92:417-424.
3. Deutsch, P. J., O. M. Rosen, and C. S. Rubin. 1982. Identification and characterization of a latent pool of insulin receptors in 3T3-L1 adipocytes. J. Biol. Chem. 257:5350-5358.
4. Tarr, J. E., F. Ray, J. H. Ward, J. P. Kubchner, and J. Kaplan. 1983. Internalization and subcellular localization of transferrin and transferrin receptors in HeLa cells. J. Cell Biol. 98:875-878.
5. Kaplan, J., and E. A. Keogh. 1982. Studies on the physiology of macrophage receptors for α-macroglobulin-protease complexes. Ann NY Acad. Sci. 421:442-456.
6. Baus, S. K., J. L. Goldstein, R. G. Anderson, and M. S. Brown. 1981. Mechanisms regulating the low density lipoprotein receptors in human fibroblast. Cell. 24:493-502.
7. Schwartz, A. L., S. E. Fridovich, and H. F. Lodish. 1982. Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hematopoietic cell line. J. Cell Biol. 92:4236-4247.
8. Greute, H. J., J. W. Slot, G. J. Stros, H. F. Lodish, and A. L. Schwartz. 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immuno- electron microscopy during receptor-mediated endocytosis. Cell. 32:277-287.
9. Kaplan, J., and E. A. Keogh. 1982. Temperature shifts induce the selective loss of alveolar-macrophage plasma membrane components. J. Cell Biol. 94:12-19.
10. Kaplan, J., F. W. Ray, and E. A. Keogh. 1981. Recognition of nucleophile-treated α-macroglobulin by the alveolar macrophage α-macroglobulin-protease complex receptor. J. Biol. Chem. 256:7105-7110.
11. Sawatzki, G., V. Arelstetter, and B. Kubanek. 1981. Isolation of mouse transferrin using salting-out chromatography on Sepharose CL-6B. Biochem. Biophys. Acta. 667:132-138.
12. McConkey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy. 29:181-186.
13. Lowdy, O. H., N. J. Rosebroough, A. L. Furr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-273.
14. Kaplan, J. 1978. Modulation of lysosomal enzyme levels in cultured cells. Biochem. Biophys. Acta. 178:376-388.
15. Edelson, P. J., and C. Erbs. 1978. Plasma membrane localization and metabolism of alkaline phosphodiesterase I in mouse peritoneal macrophages. J. Exp. Med. 147:77-92.
16. Bergmeyer, H. U., E. Bernt, and B. Hess. 1965. In Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 736-741.
17. Kaplan, J., and M. N. Nielsen. 1979. Analysis of macrophage surface receptors: internalization of α-macroglobulin trypsin complexes by rabbit alveolar macrophages. J. Biol. Chem. 254:7329-7335.
18. Addanks, S., J. F. Sotos, and P. D. Reaick. 1966. Rapid determination of picoenzyme quantities of ATP with a liquid scintillation counter. Anal. Biochem. 14:261-264.
19. Wiley, H. S., and D. D. Cunningham. 1962. The endocytic rate constant. A cellular parameter for quantitating receptor-mediated endocytosis. J. Biol. Chem. 237:4222-4229.
20. Scott, R. E., R. G. Perkins, M. A. Zschunke, B. J. Hoerl, and P. B. Maercklein. 1979. Fractionation of 125I-labeled asialo-fetuin by isolated rat hepatocytes. Biochem. Biophys. Acta. 599:73-84.
21. Kaplan, J., and M. N. Nielsen. 1978. Pinocytic activity of rabbit alveolar macrophages in vitro. J. Reticuloendothel. Soc. 24:673-685.
22. Gonzalez-Noriega, A., and W. S. Sty. 1978. Concanavalin A-mediated uptake of enzymes by fibroblasts. Biochem. Biophys. Res. Commun. 85:174-182.
23. Tollenshag, H., T. Berg, M. Nilsson, and K. R. Norum. 1977. Uptake and degradation of125I-labeled asialo-fetuin by isolated rat hepatocytes. Biochem. Biophys. Acta. 499:73-84.
24. Kaplan, J., and E. A. Keogh. 1981. Analysis of the effect of amine in inhibition of receptor-mediated and fluid-phase pinocytosis in rabbit alveolar macrophages. Cell. 24:925-932.
26. Dunn, W. A., A. L. Hubbard, and N. N. Aronson. 1980. Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of 125I-asialofetuin by the perfused rat liver. J. Biol. Chem. 255:5971-5978.

27. Cailloway, C. J., G. E. Dean, M. Marsh, G. Rodnick, and I. Mellman. 1983. Acidification of macrophage and fibroblast endocytic vesicles in vitro. Proc. Natl. Acad. Sci. USA. 80:3334-3338.

28. Tantle, D. W., E. Wu, and W. W. Webb. 1982. Enhanced molecular diffusibility in muscle membrane blebs: release of lateral constraints. J. Cell Biol. 92:207-212.

29. Power, J. A., J. W. Harris, and D. F. Bainton. 1977. Lipid peroxidation and morphological changes in mammalian cells treated with the glutathione oxidant, diamide. Exp. Cell Res. 105:455-460.

30. Scott, R. E., and P. B. Maercklein. 1979. Plasma membrane vesiculation in 3T3 and SV3T3 cells. II. Factors affecting the process of vesiculation. J. Cell Sci. 35:245-252.

31. Brachet, J., E. Baltus, A. De Schutter-Pays, J. Hanocq-Quertier, E. Hubert, and G. Steinert. 1975. Induction of maturation (meiosis) in Xenopus laevis oocytes by three organomercurials. Proc. Natl. Acad. Sci. USA. 72:1574-1578.

32. Kono, T., F. W. Robisson, T. L. Blevins, and O. Ezaki. 1982. Evidence that translocation of the glucose transport activity is the major mechanism of insulin action on glucose transport in fat cells. J. Biol. Chem. 257:10942-10947.

33. Bindoli, A., and S. Fleischer. 1983. Induced Ca²⁺ release in skeletal muscle sarcoplasmic reticulum by sulphydryl reagents and chlorpromazine. Arch. Biochem. Biophys. 221:458-466.

34. Wasserman, W. J., and Y. Masui. 1975. Initiation of meiotic maturation in Xenopus laevis oocytes by the combination of divalent cations and ionophore A23187. J. Exp. Zool. 193:369-375.

35. Douglas, W. W. 1978. Stimulus secretion coupling: variations on the theme of calcium-activated exocytosis involving cellular and extracellular sources of calcium. Ciba Found. Symp. 54:61-90.

36. Buys, S. S., E. A. Keogh, and J. Kaplan. 1984. Fusion of intracellular membrane pools with cell surface of macrophages stimulated by phorbol esters and calcium ionophores. Cell. 38:569-576.

37. Webb, J. L. 1966. Enzyme and Metabolic Inhibitors. Vol. III. Academic Press, Inc. New York. 535-651.

38. Wiley, H. S. and J. Kaplan. 1984. Epidermal growth factor rapidly induces a redistribution of transferrin receptor pools in human fibroblasts. Proc. Natl. Acad. Sci. USA. 81:7456-7460.

39. Petty, H. R., D. G. Hafeman, and H. M. McConnell. 1981. Disappearance of macrophage surface folds after antibody-dependent phagocytosis. J. Cell Biol. 89:223-229.