

Inhibition of Receptor-mediated But Not Fluid-Phase Endocytosis in Polymorphonuclear Leukocytes

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ABSTRACT We have found that hypertonic medium inhibited the receptor-mediated uptake of the chemotactic peptide *N*-formylnorleucylleucylphenylalanine without affecting fluid-phase endocytosis by polymorphonuclear leukocytes (PMNs). Morphological and biochemical evidence demonstrated that cells in hypertonic medium did not accumulate peptide in a receptor-mediated manner. However, the cells continued to form endosomes containing fluid-phase markers. Furthermore, the content of these endosomes was processed normally, i.e., both digested and intact material were released into the medium. The inhibition of receptor-mediated uptake was a function of the tonicity. Partial inhibition occurred in 0.45 and 0.6 osmolar medium and maximal inhibition occurred in 0.75 osmolar medium. The inhibition was independent of the solute used to increase the tonicity: sodium chloride, sucrose, and lactose all inhibited uptake to similar extents. Hypertonic medium had little effect on saturable peptide binding. However, it did prevent the clustering of surface molecules as indicated by the inhibition of capping of fluorescent concanavalin A. In addition, hypertonic medium prevented the peptide-stimulated increase in cytosolic calcium levels as measured by quin 2 fluorescence. The tonicity dependence of the inhibition of quin 2 fluorescence paralleled the inhibition of receptor-mediated uptake.

The signals and mechanisms involved in the initiation of both receptor-mediated and fluid-phase endocytosis have been studied for a number of years (12). The factors controlling the initiation of endocytosis vary depending on the cell type and specific endocytic pathway being studied. One point of confusion is the relationship between receptor-mediated uptake of ligands and the uptake of fluid-phase markers. In some cases, the addition of ligand stimulates the uptake of fluid-phase markers while in other cases, there is no change in the rate of fluid accumulation by the addition of ligand (1, 3, 11, 13). Interpretation of this data is further confused by the fact that it is unknown whether all uptake occurs in one class of vesicles or whether there are separate pathways of uptake. Marsh and Helenius (7) have argued that all fluid-phase uptake in baby hamster kidney cells can be accounted for by the volume included in coated vesicles while Haigler et al. (4) found that in A-431 cells the fluid-phase marker, horseradish peroxidase, and the specific ligand, epidermal growth factor, appeared within separate, morphologically distinct vesicles. This was seen even when the markers were incubated with the cells for as short a time as 30 s (4).

One approach to examining the question of the uptake and subsequent fate of fluid markers and specific ligands is to develop conditions which selectively alter one or the other endocytic pathway. However, treatments which reduce the receptor-mediated accumulation of ligand often produce a decrease in fluid-phase endocytosis (5, 6). We now report that hypertonic medium selectively inhibits receptor-mediated uptake without affecting fluid-phase endocytosis. Our studies show that in polymorphonuclear leukocytes (PMNs),¹ the receptor-mediated accumulation of the chemotactic peptide *N*-formylnorleucylleucylphenylalanine (f-NleLeuPhe) is inhibited by 0.75 osmolar medium, while fluid-phase endocytosis is not affected. Endosomes containing fluid-phase mark-

¹ *Abbreviations used in this paper:* Con A, concanavalin A; FITC, fluorescein isothiocyanate; f-NleLeuPhe, *N*-formylnorleucylleucylphenylalanine; HBSS, Hanks' balanced salt solution; hexapeptide, *N*-formyl-NleLeuPheNleTyrLys; hNaCl, HBSS plus 0.225 M NaCl; hsucrose, HBSS plus 0.45 M sucrose; Hy, hemocyanin; K_d , dissociation constant; PMN, polymorphonuclear leukocytes; RBCs, erythrocytes; TCA, trichloroacetic acid; TMR, tetramethylrhodamine isothiocyanate.

ers continue to form and deliver these markers to intracellular compartments where digestion occurs. This inhibition is not due to an effect on the binding of peptide to its receptor. In addition, we show that 0.75 osmolar medium completely prevents the peptide-stimulated increase in cytosolic calcium levels as measured by the fluorescent probe, quin 2. The inhibition of the peptide stimulation of quin 2 shows a similar dependence on the tonicity as the inhibition of receptor-mediated uptake with maximal inhibition occurring in 0.75 osmolar medium.

MATERIALS AND METHODS

Cells: Rabbit peritoneal exudate PMNs were collected 4 h after injection of 0.1 g of shellfish glycogen (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) in 250 ml of 0.9% saline. Contaminating erythrocytes (RBCs), if present, were lysed by a brief (60-s) treatment with hypotonic (0.18%) saline. The cells were washed twice with 0.9% saline and resuspended in Hanks' balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY) buffered with 2.4 mg/ml of HEPES (Sigma Chemical Co., St. Louis, MO) at a concentration of 3.3×10^6 cells/ml. 3 ml of the cell suspension was placed in 60 × 15-mm petri dishes (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) and the cells were allowed to settle for 15 min at 23°C. Dishes were checked with an inverted microscope to ensure that an even monolayer had formed.

Human PMNs were obtained from blood by venipuncture. The blood was mixed with 0.6% dextran 70 and 0.02 M sodium citrate in saline (final concentrations). The RBCs were allowed to settle, the plasma layer was removed, and the cells were pelleted at 400 g for 10 min. Contaminating RBCs were lysed by exposure to 0.18% saline for 30 s. Cells were washed and resuspended in Ca^{++} - and Mg^{++} -free HBSS, layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), and spun 20 min at 600 g. The cell pellet was resuspended and washed twice in HBSS without phenol red.

Media: The tonicity of the media was adjusted to the final desired osmolarity by the addition of the appropriate amount of sucrose, lactose, or sodium chloride. Cells were pretreated for 10 min at 37°C with the hypertonic medium prior to the start of an experiment.

Peptide Uptake and Release: Cells were incubated for varying times with [^3H]f-NleLeuPhe (New England Nuclear, Boston, MA; specific activity 11 Ci/mM) at 37°C and then washed for 10 min at 4°C. This has previously been shown to remove >95% of the receptor-bound peptide (13). The peptide remaining cell associated after this wash is the "total uptake."

By varying the concentration of peptide, we could follow either receptor-mediated or fluid-phase uptake. Rabbit peritoneal PMNs have a receptor for f-NleLeuPhe which has a dissociation constant, K_d , of about 2×10^{-8} M. From a dose-response curve of f-NleLeuPhe uptake, we have determined the percentage of the total uptake which occurred via a receptor-mediated process at each concentration of peptide (2). Both total uptake and nonsaturable uptake (^3H -peptide uptake in the presence of an excess of unlabeled peptide [1,000 times the K_d concentration]) were measured. Receptor-mediated uptake was calculated by subtracting nonsaturable uptake from total uptake. At concentrations of peptide $<3 \times 10^{-8}$ M, the majority of the uptake is via a receptor-mediated process, specifically, at 2×10^{-8} M, 80% of the uptake occurs in a saturable manner. The accumulation of peptide at concentrations above $\sim 3 \times 10^{-8}$ M is proportional to the concentration of peptide present in the extracellular medium. At these high concentrations, peptide acts as a fluid-phase marker. At 10^{-5} peptide, only 1% of the counts accumulated could be accounted for by a saturable process. These experimentally derived values are consistent with the following calculation which estimates the proportion of total uptake of peptide that occurs in the fluid-phase when cells are incubated with 10^{-5} M f-NleLeuPhe. At 10^{-5} M f-NleLeuPhe (1,000 times the K_d concentration), all of the receptors should be occupied, i.e., approximately two times the number occupied at 2×10^{-8} M (K_d). Therefore, the amount of peptide taken up in receptor-mediated manner would be expected to be twice that observed at 2×10^{-8} M. In Fig. 1, where the specific activity of the 10^{-5} M peptide was 0.01 times that of the 2×10^{-8} M peptide, the receptor-mediated uptake of radiolabeled peptide at 10^{-5} M f-NleLeuPhe would be expected to amount to 2% of the counts taken up at 2×10^{-8} M. In this experiment, the total counts taken up were approximately the same for cells in 10^{-5} M and 2×10^{-8} M peptide. Thus 2% of the total uptake at 10^{-5} M appears to be via a receptor-mediated process and the remaining 98% must occur via the fluid phase.

Fluid uptake was also measured by the uptake of [^{14}C]sucrose (New England Nuclear) and [^{125}I]hemocyanin (Hy) (iodinated as described previously for bovine serum albumin [2]). Hy degradation was monitored by precipitation with 10% trichloroacetic acid (TCA).

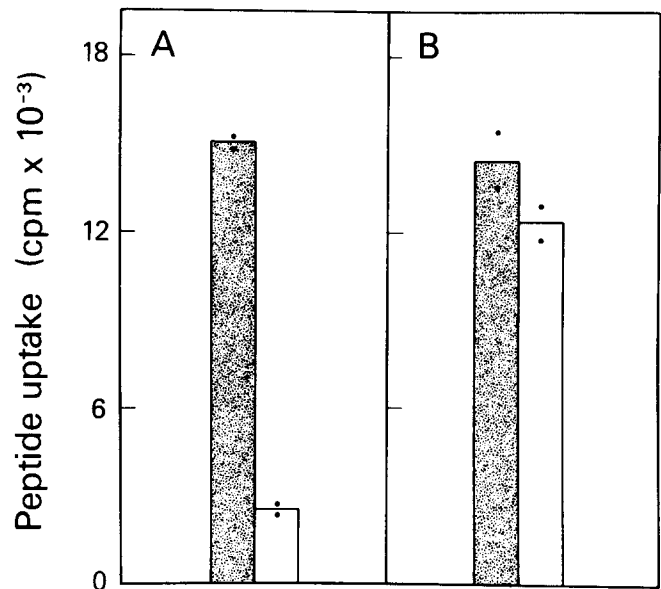


FIGURE 1 Effects of hypertonic medium on receptor-mediated and fluid-phase uptake of f-NleLeuPhe. PMNs were incubated for 30 min at 37°C in either HBSS or hNaCl containing (A) 2×10^{-8} M [^3H]f-NleLeuPhe to follow receptor-mediated uptake or (B) 10^{-5} M [^3H]f-NleLeuPhe (at 0.01 times normal specific activity) to follow fluid-phase uptake. The dark bars are cells in HBSS; the white bars are cells in hNaCl.

To measure release of the cell-associated radioactivity, cells were incubated with [^3H]f-NleLeuPhe or [^{125}I]Hy, washed, and were then returned to 37°C in HBSS for various times. The medium was removed and counted. The radioactivity remaining with the cells was also counted.

Peptide Digestion: Peptide digestion was assayed by partitioning into ethyl acetate. The aqueous medium containing released cell-associated radioactivity was brought to pH 2 with 1 N HCl, and an equal volume of ethyl acetate was added. After thorough mixing, the solution was allowed to separate into two phases. The intact f-NleLeuPhe, having a blocked amino group, partitions into ethyl acetate and the digestion products (any molecule with a free amino group will be protonated at pH 2) remain in the aqueous phase. The radioactivity (the peptide is labeled with [^3H]phenylalanine) in each of the phases was then counted. This analysis of peptide digestion was confirmed using thin layer chromatography. Hypertonic medium (regardless of solute used to increase the tonicity) had no effect on the partitioning assay.

Binding Studies: Each dish containing 10^7 cells was incubated in 1.0 ml of HBSS (or the appropriate hypertonic solution) with 10^{-7} M [^3H]f-NleLeuPhe at 4°C. After incubation, this medium was aspirated and the dish washed quickly (~6 s) and vigorously in two baths of 4°C saline of the appropriate tonicity. Cell-associated radioactivity was counted. In all experiments the dishes were monitored visually for cell loss.

Fluorescent Reagents: The chemotactic peptide N-formyl-NleLeuPheNleTyrLys (hexapeptide) (Sigma Chemical Co.), was conjugated to tetramethylrhodamine isothiocyanate (TMR) (Research Organics Inc.) following the procedure of Niedel et al. (9). Briefly, TMR (10 mM) was reacted with 2.5 mM hexapeptide in dry dimethylformamide containing 1% triethylamine for 18 h at 22°C. The product TMR-hexapeptide was purified by thin layer chromatography in chloroform, methanol, and triethylamine (5:2:1 by volume) (TMR-hexapeptide, $R_f = 0.3$; unconjugated hexapeptide, $R_f = 0.15$). Fluorescein isothiocyanate-Con A (FITC-Con A) (Sigma Chemical Co.) was used at a concentration of 500 $\mu\text{g}/\text{ml}$ and FITC-dextran (Sigma Chemical Co.) was used at a concentration of 5 mg/ml.

Microscopy: Cells were viewed by phase-contrast and fluorescence microscopy equipped with SIT camera. Video output was recorded with a NEC video recorder. The images were photographed directly from the monitor.

Quin 2 Assay: Human cells (2×10^7 cells/ml) in HBSS without phenol red containing 0.1% ovalbumin and 20 μM acetoxymethyl ester of quin 2 (Amersham Corp., Arlington Heights, IL) at pH 7.4 were incubated for 20 min in the dark at 37°C followed by a 20-min incubation at room temperature. The cells were washed twice and resuspended at 10^7 cells/ml in HBSS without phenol red containing 0.1% ovalbumin and kept on ice until used. For each assay 10^7 cells were pelleted and resuspended in 2 ml of medium of the

appropriate tonicity prewarmed to 37°C. The cells were incubated at 37°C for 10 min before 10^{-7} M *n*-formyl-MetLeuPhe was added. The changes in fluorescence were measured in a stirred cuvette in a Perkin-Elmer spectrofluorometer (excitation at 339 nm and emission at 492 nm). Intracellular calcium concentration was calculated using the following equation:

$$[Ca^{++}]_{in} = 1.15 \times 10^{-7} \frac{(F - F_{af}) - (F_{min} - F_{min af})}{(F_{max} - F_{max af}) - (F - F_{af})}$$

in which 1.15×10^{-7} M = K_d for Ca^{++} binding to quin 2; F = fluorescent signal in response to stimulation of cells loaded with quin 2; F_{max} = fluorescent signal after cells are lysed with 0.2% Triton X-100; F_{min} = fluorescent signal in lysed cells plus 1 mM EGTA; F_{af} , $F_{max af}$, and $F_{min af}$ = autofluorescent measurements in cells which have not been loaded with quin 2 (intact cells, lysed cells, and lysed cells plus 1 mM EGTA, respectively).

RESULTS

Hypertonic Medium Inhibits Receptor-mediated But Not Fluid-Phase Uptake

The chemotactic peptide, f-NleLeuPhe, is taken up by peritoneal PMNs in both a receptor-mediated and fluid-phase manner (13). By varying the concentration of peptide, we can follow either receptor-mediated or fluid-phase uptake (see Materials and Methods). PMNs have a receptor for f-NleLeuPhe which has a dissociation constant, K_d , of $\sim 2 \times 10^{-8}$ M. At 2×10^{-8} M, $\sim 80\%$ of the peptide uptake is saturable, i.e., can be competed for with a high concentration of unlabeled peptide. Thus, 80% of the uptake at 2×10^{-8} M is via a receptor-mediated process. At 10^{-5} M (1,000 times the K_d), $\sim 98\%$ of the peptide uptake is accumulated via uptake of bulk fluid. We have used 2×10^{-8} M f-NleLeuPhe as an indicator of receptor-mediated uptake and 10^{-5} M f-NleLeuPhe as a fluid-phase marker. Placing the cells in hypertonic medium inhibited the receptor-mediated accumulation of f-NleLeuPhe without affecting the fluid-phase uptake of peptide (Fig. 1). The persistence of fluid-phase uptake in hypertonic medium was confirmed using another fluid-phase marker, [14 C]sucrose. Cells in hypertonic medium accumulated the same amount of [14 C]sucrose as did control cells (Fig. 2).

Morphological Examination of Effects of Hypertonic Medium

To examine morphologically the effect of hypertonic medium on pinocytosis, we followed the uptake of the fluorescent

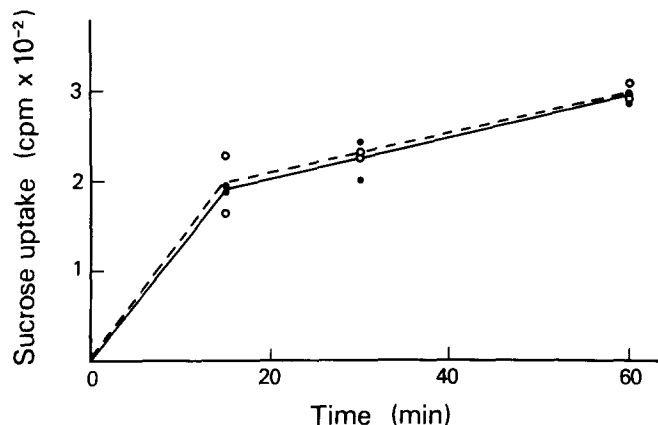


FIGURE 2 Effect of hypertonic medium on the uptake of the fluid-phase marker, [14 C]sucrose. PMNs were incubated with [14 C]sucrose (12 μ Ci/ml) and 2×10^{-8} M unlabeled f-NleLeuPhe for various times at 37°C in HBSS (○) or in hNaCl (●).

hexapeptide labeled with tetramethylrhodamine (TMR-hexapeptide). The hexapeptide has been shown to compete for the same receptor as f-NleLeuPhe (9). PMNs, exposed to 2×10^{-9} M TMR-hexapeptide at 37°C, accumulated fluorescence within cytoplasmic organelles (both endocytic vesicles and phase-dense granules) which exhibited saltatory motion (Fig. 3, *a* and *b*). The accumulation of fluorescence was due to receptor-mediated uptake since it could be competitively inhibited by an excess of unlabeled f-NleLeuPhe (Fig. 3, *c* and *d*). When PMNs were exposed to 2×10^{-9} M TMR-hexapeptide in 0.75 osmolar medium (HBSS plus 0.225 M NaCl), the accumulation of fluorescence was dramatically inhibited (Fig. 3, *e* and *f*). However, hypertonic medium had no effect on the accumulation of a fluorescent fluid-phase marker, FITC-dextran (Fig. 3, *g-j*). Similarly hypertonic medium had no effect on the accumulation of 10^{-5} M TMR-hexapeptide. At this concentration, peptide accumulation occurs primarily in a fluid-phase manner. Thus, hypertonic medium inhibited the receptor-mediated internalization of peptide but did not prevent the formation of endosomes involved in the uptake of fluid markers.

Characterization of Inhibition

The extent of the inhibition of receptor-mediated uptake depended on the tonicity. Partial inhibition occurred in 0.45 and 0.6 osmolar medium, while maximal inhibition occurred in 0.75 osmolar medium (2.5 times normal tonicity) (Fig. 4). The inhibition was independent of the solute used to increase the tonicity. Increasing the tonicity with sodium chloride, sucrose, or lactose inhibited uptake to a similar extent. The inhibition of receptor-mediated processes did not appear to occur at the level of receptor binding since incubation of cells 30 min in hypertonic medium at 37°C had little effect on subsequent peptide binding assayed at 4°C in hypertonic medium. In medium containing 0.45 M sucrose, the amount of saturably bound peptide at 2×10^{-8} M was 1.24 ± 0.33 times that bound to cells incubated in control HBSS (mean \pm SD, $n = 8$). With addition of 0.225 M NaCl to HBSS, the binding was 0.96 ± 0.13 ($n = 3$) of control levels.

The inhibition was rapid; maximal inhibition of uptake occurred within 5 min of placing cells in hypertonic medium. The effect was also rapidly reversible. Uptake returned to control levels within 5 min of returning the cells to isotonic medium, i.e., the slopes of the uptake curves were the same 5 min after return to control medium (Fig. 5). The reversibility of the inhibition of hypertonic medium on the receptor-mediated accumulation was also followed morphologically. When PMNs were removed from hypertonic medium and placed in isotonic medium, they accumulated TMR-hexapeptide in a receptor-mediated manner.

Hypertonic Medium Inhibits the Capping of Con A

The fluorescent signal from the TMR-hexapeptide was too low to determine whether hypertonicity had any effect on the clustering of the peptide-receptor complex. Thus, to examine the effect of hypertonic medium on the aggregation and distribution of a cell surface marker, we followed the capping of Con A. Cells were incubated with FITC-Con A in control or hypertonic medium for 15 min at 37°C. While $>80\%$ of cells in control medium capped the FITC-Con A, $<4\%$ of the cells in hypertonic medium capped the FITC-Con A (Fig.

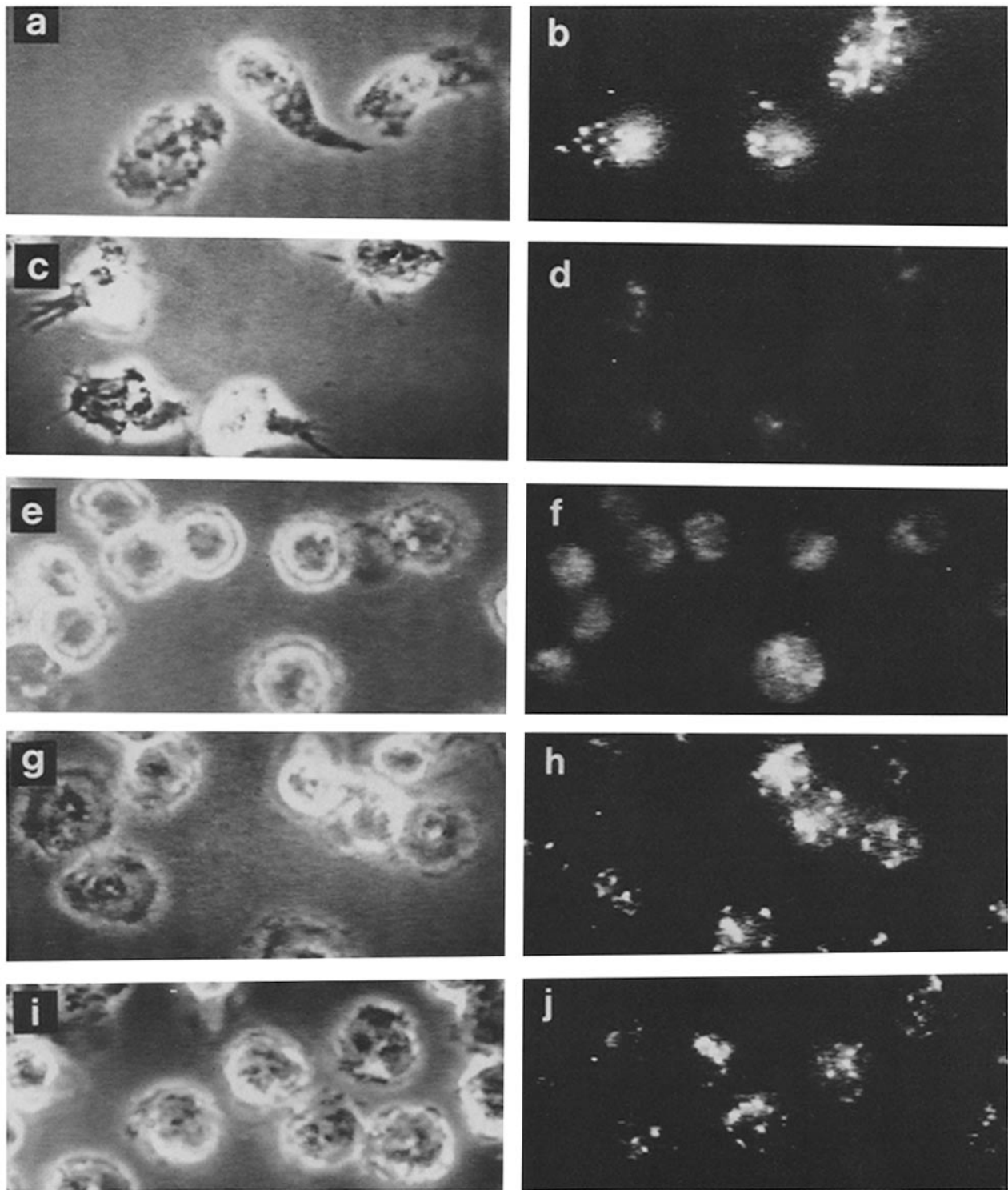


FIGURE 3 Morphological examination of the effect of hypertonicity on receptor-mediated and fluid-phase endocytosis. PMNs were incubated for 30 min at 37°C with different fluorescent reagents in either HBSS or hNaCl. Living cells were viewed by phase-contrast microscopy (a, c, e, g, and i) or fluorescence microscopy (b, d, f, h, and j). To monitor receptor-mediated uptake, cells were incubated in HBSS with 2×10^{-9} M TMR-hexapeptide (a and b). To document that the accumulation of the fluorescence observed in b was due to a saturable process, cells were incubated in HBSS with 2×10^{-9} M TMR-hexapeptide plus 10^{-5} M f-NleLeuPhe to compete for receptor binding (c and d). To evaluate receptor-mediated uptake in hypertonic medium, cells were incubated in 2×10^{-9} M TMR-hexapeptide in hNaCl (e and f). To monitor fluid phase uptake, cells were incubated in FITC-dextran in HBSS (g and h) or in hNaCl (i and j).

6 and Table I). The cells in hypertonic medium showed a diffuse pattern of fluorescence even after 60 min at 37°C. The inhibition of capping was reversible. Cells which had bound

FITC-Con A in hypertonic NaCl were pelleted and resuspended in HBSS in the absence of FITC-Con A. The FITC-Con A that was bound to the cells in hypertonic medium was

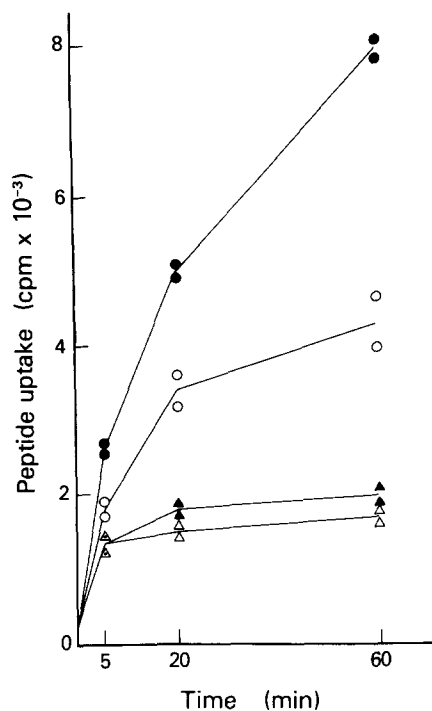


FIGURE 4 Effect of increasing tonicity on the receptor-mediated uptake of peptide. PMNs were incubated with 2×10^{-8} M [3 H]-f-NleLeuPhe at 37°C for various times in HBSS (●) or HBSS plus different concentrations of lactose (0.3 M (○), 0.45 M (▲), or 0.6 M (△)).

now capped (Table I). This result also demonstrates that the inability to visualize capping in hypertonic medium was not due to insufficient binding of FITC-Con A to the cell surface.

Characterization of Processing of Endocytosed Material in Hypertonic Medium

In Fig. 3, we showed that hypertonic medium did not prevent the formation of endosomes containing fluid-phase markers. To assess whether there was any alteration of the intracellular fate of this endocytosed material, we examined the processing of this cell-associated material. After PMNs had accumulated peptide in a fluid-phase manner, peptide was removed and the cells were incubated in hypertonic medium at 37°C . As can be seen in Fig. 7a, hypertonic medium did not prevent the release of either intact or digested peptide. In fact, the proportion of f-NleLeuPhe released as digested peptide was slightly increased in hypertonic medium. We obtained similar results following the processing of another fluid-phase marker, [125 I]Hy. The uptake of [125 I]Hy and the release of digested products (TCA-soluble radioactivity) and intact material (TCA-precipitable radioactivity) in hypertonic medium was similar to that seen in control cells (Fig. 8). Thus, in hypertonic medium, endosomes containing fluid-phase markers appear to be processed normally.

Although hypertonic medium inhibited the continuing accumulation of peptide in a receptor-mediated manner, there was a small amount of peptide accumulation which reached a plateau within 5 min (Figs. 4 and 5). Upon further incubation in the absence of peptide, the majority of this cell-associated peptide was released from the cells. The amount of intact peptide released from cells in hypertonic medium was

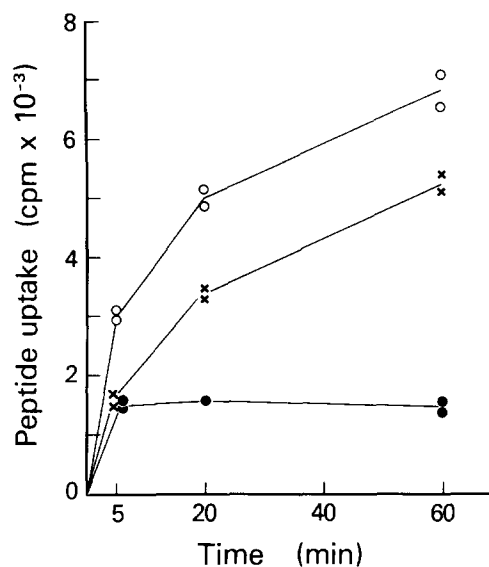


FIGURE 5 Reversal of the inhibition by hypertonic medium on receptor-mediated uptake. PMNs were pretreated with HBSS or HBSS plus 0.45 M sucrose (hsucrose), then uptake was measured in HBSS or hsucrose. Pretreatment and uptake in HBSS (○); pretreatment and uptake in hsucrose (●); pretreatment in hsucrose, uptake in HBSS (×).

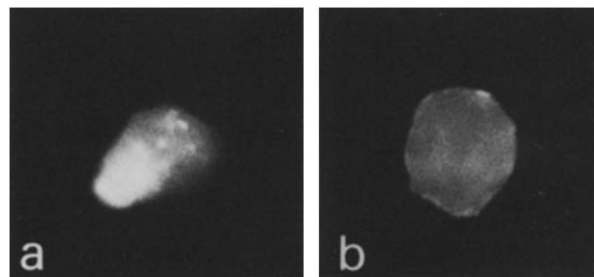


FIGURE 6 Effect of hypertonicity on capping of Con A. Cells were incubated for 15 min at 37°C in FITC-Con A in HBSS (a) or in hNaCl (b).

TABLE I. Effect of Hypertonicity on Con A Capping

Media	Cells capped in 15 min at 37°C	Cells capped after being washed and resuspended in HBSS for 15 min at 37°C
	%	%
HBSS	$84 \pm 3.3; n = 3^*$	$77 \pm 7; n = 2$
hNaCl*	$3.7 \pm 5.3; n = 3$	$60 \pm 9; n = 2$

* n, the number of different experiments analyzed. 100 cells per sample were scored. The mean \pm the standard deviation is given.

* hNaCl, HBSS plus 0.225 M NaCl.

the same as that seen in control cells (Fig. 7b). The kinetics of the uptake and release of the pool of intact peptide were similar to that seen in control cells. However, in contrast to that seen with fluid-phase markers, hypertonicity dramatically decreased the amount of digested peptide released. Thus, hypertonic medium prevented receptor-mediated peptide uptake and delivery of peptide into a compartment where digestion occurs but did not prevent the filling of a compartment from which peptide is subsequently released intact.

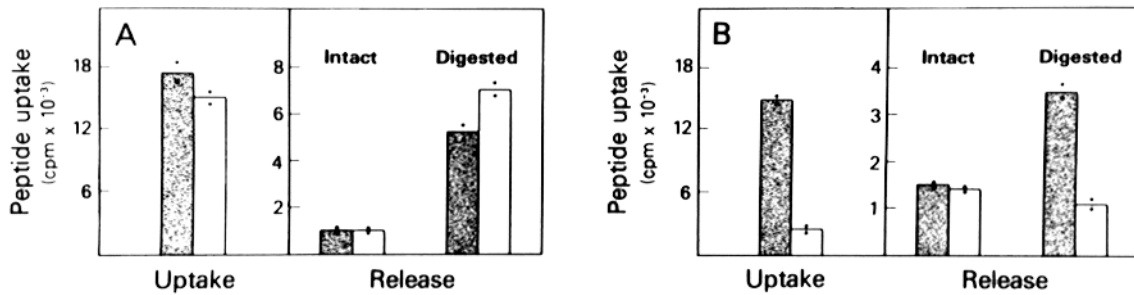


FIGURE 7 Effect of hypertonic medium on processing of endocytosed peptide. (A) To examine the effect on the processing of peptide accumulated in a fluid-phase manner, PMNs were incubated with 10^{-5} M [3 H]f-NleLeuPhe for 30 min at 37°C in HBSS (filled bars) or in hNaCl (open bars). The cells were washed at 4°C and returned to 37°C in HBSS or hNaCl. After 30 min, the medium was removed and analyzed for the presence of digested and intact peptide. (B) To examine the processing of peptide accumulated in a receptor-mediated manner, PMNs were incubated with 2×10^{-8} M [3 H]f-NleLeuPhe for 30 min at 37°C in HBSS or in hNaCl. Cells were treated as above and the medium was analyzed for the presence of digested and intact peptide.

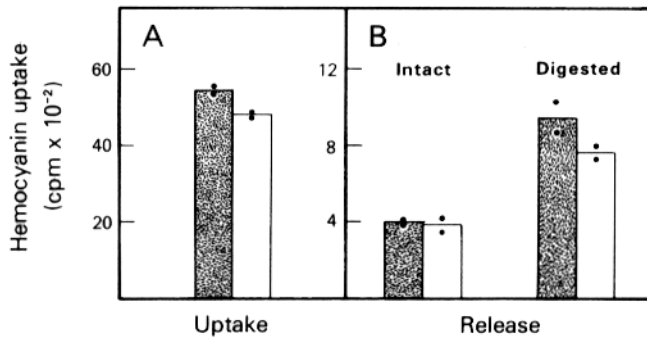


FIGURE 8 Effect of hypertonic medium on the uptake and processing of [125 I]Hy. (A) Uptake: PMNs were incubated for 30 min at 37°C with [125 I]Hy (6.5 μ Ci/ml) in HBSS (dark bars) or in hNaCl (white bars). (B) Release: After a 30-min uptake at 37°C, cells were washed at 4°C and returned to 37°C in HBSS or in hNaCl. After 30 min, the medium was removed and analyzed for the presence of TCA-precipitable (intact) and TCA-soluble (digested) radioactivity.

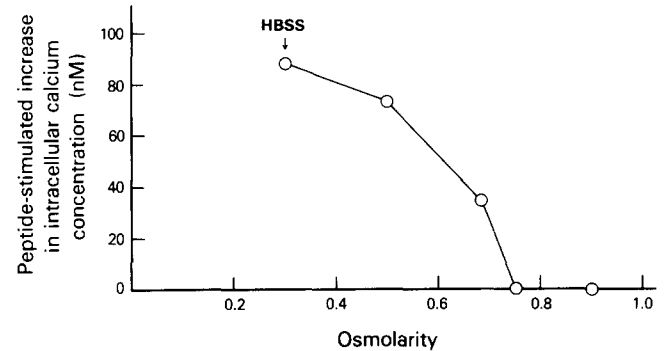


FIGURE 9 Effect of increasing tonicity of peptide-stimulated increase in cytosolic calcium concentration. The fluorescent signal from quin 2 containing PMNs in media of varying tonicity was recorded both before and after addition of 10^{-7} M *n*-formyl-Met-LeuPhe. The maximum stimulation of intracellular calcium concentration is plotted as a function of osmolarity. The tonicity of the medium was increased by the addition of NaCl to HBSS. Determination of intracellular calcium concentration is described in Materials and Methods.

Effect of Hypertonicity on Peptide-stimulated Increase in Cytosolic Calcium

Chemotactic factors induce a rapid, transient increase in cytosolic calcium levels which can be measured with the fluorescent probe, quin 2 (10). We have asked whether addition of peptide would induce an increase in cytosolic calcium in cells in hypertonic medium. PMNs loaded with quin 2 in HBSS were switched to media of varying tonicities for 10 min at 37°C prior to peptide addition. Raising the osmolarity of the medium to 0.75 osmolar completely prevented the quin 2 signal upon addition of 10^{-7} M *n*-formyl-MetLeuPhe (Fig. 9). The inhibition of the peptide-stimulation of quin 2 increased as the tonicity of the medium was increased and paralleled the inhibition of receptor-mediated uptake (Fig. 9).

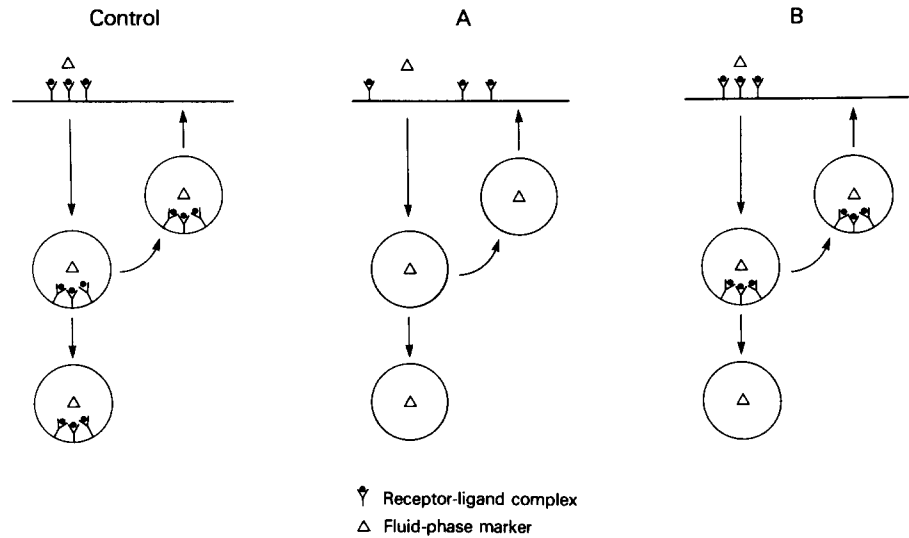
DISCUSSION

We have found that hypertonic medium inhibited receptor-mediated but not fluid-phase endocytosis by PMNs. The effects of hypertonicity were documented biochemically and morphologically using [3 H]f-NleLeuPhe and TMR-hexapeptide as markers of receptor-mediated uptake and [14 C]sucrose, [125 I]Hy, FITC-dextran, and high concentrations of [3 H]f-NleLeuPhe as markers of the fluid phase. We showed that endosomes that contain fluid-phase markers continue to form in hypertonic medium and appear to be processed normally by the cell, i.e., some of the material taken up was released

back to the medium intact while most was digested and then released. Hypertonic medium prevented the receptor-mediated uptake and delivery of peptide into a compartment where digestion occurs but did not prevent the filling of a compartment from which peptide is subsequently released intact. The extent of the inhibition increased with increasing tonicity with maximal inhibition occurring at 0.75 osmolar. The inhibition was independent of the solute used to increase the tonicity; sodium chloride, sucrose, and lactose all gave similar results. The inhibition was rapid and rapidly reversible. The reversibility of the effect and the persistence of fluid-phase uptake indicated that the inhibition was not due to nonspecific toxicity of the hypertonic medium.

Hypertonic medium appears to be unusual in its ability to rapidly and reversibly separate fluid-phase and receptor-mediated endocytosis by selectively inhibiting receptor-mediated uptake. Most conditions reported to inhibit receptor-mediated uptake also inhibit fluid-phase uptake. Larkin et al. (6) found that depletion of intracellular potassium results in the inhibition of the receptor-mediated uptake of both low density lipoprotein and epidermal growth factor and the fluid-phase uptake of horseradish peroxidase in fibroblasts. This treatment also reduced the number of coated pits (6). Kaplan and Keogh (5) have shown that in macrophages, the addition of amines decreased fluid-phase pinocytosis as well as the recep-

FIGURE 10 Proposed models for the mechanism of inhibition of receptor-mediated uptake by hypertonic medium. (A) Hypertonic medium prevents the internalization of receptor-ligand complexes. This could result from inhibition of surface receptor clustering. (B) Hypertonic medium allows receptor-ligand internalization but prevents movement of receptor-ligand complexes from a primary recycling endosome.



tor-mediated uptake of α_2 -macroglobulin. The weak bases were shown to decrease cell surface receptor number and to inhibit α_2 -macroglobulin accumulation. The mechanism of inhibition of fluid-phase pinocytosis was unclear (5).

To determine if hypertonic medium inhibited transduction of peptide-induced signals, we examined the peptide-stimulated increase in cytosolic calcium. This is an early response to peptide binding. Using the fluorescent calcium probe, quin 2, we found that incubating cells in 0.75 osmolar medium completely prevented the peptide-stimulated increase in cytosolic calcium levels. The extent of this inhibition showed a similar dependence on tonicity as that seen for the inhibition of receptor-mediated uptake. It is unknown whether the inhibition of Ca^{++} mobilization and receptor-mediated uptake are due to the loss of a common intermediate coupling peptide-receptor binding to cellular responses. However, the similarity in the dose dependence of the two phenomena suggests this as a possibility.

Our data are consistent with the models pictured in Fig. 10. In model A, hypertonicity prevents the internalization of surface receptor-bound peptide by preventing receptor clustering into forming endosomes. Fluid-phase material continues to be internalized and delivered to the digestion compartment. Unclustered receptors would only inefficiently facilitate ligand internalization. In support of this hypothesis, we have shown that hypertonic medium inhibits the surface aggregation of Con A.

In model B, hypertonic media allows receptor-ligand internalization but prevents their movement from a primary recycling endosome. In this model, both fluid-phase markers and peptide-receptor complexes are internalized in hypertonic medium into a reversible pinocytic compartment (recycling endosome pool). Fluid-phase material can be delivered to the lysosomal compartment where digestion occurs but the peptide-receptor complex remains in a reversible pinocytic compartment which is in steady-state with the cell surface. Mellman et al. (8) have presented a similar model to explain the recycling of the Fc receptor. They suggest that the state of the receptor aggregation may determine whether an internalized receptor remains in an endosome pool from which it can be recycled back to the cell surface or is removed from this pool and delivered to the lysosomes.

A third model would hypothesize two separate pathways of uptake; one for fluid-phase and a second for receptor-media-

ted uptake. In this case, hypertonicity would selectively inhibit the pathway for receptor-mediated uptake.

Whichever model turns out to be right, the relationship between the binding and internalization of receptor-ligand complexes and the uptake of soluble markers is of interest in understanding the factors involved in the initiation of endocytosis as well as those factors important in the sorting of constituents within endosomal (pre-lysosomal) compartments. Hypertonic medium will be a useful tool with which to investigate the initial steps involved in the endocytic pathway.

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