Concomitant Increase in Putrescine Incorporation with Transferrin Uptake into Rat Reticulocytes

Shinsuke KATOH,* Yoshie ISHII, Yumiko OHBA, Hiroyuki KOHNO and Yasuhito OHKUBO

Department of Radiopharmacy, Tohoku College of Pharmacy, 4–4–1, Komatsushima, Aoba-ku, Sendai 981, Japan. *Received July 29, 1991*

Concomitant increase in putrescine incorporation with transferrin uptake into rat reticulocytes was found. [^{14}C]Putrescine incorporation occurred in the presence of transferrin at 37 °C. The subcellular distribution of incorporated [^{14}C]putrescine showed that the incorporation in the plasma membrane time dependently increased. Both ^{125}I-labeled transferrin uptake and [^{14}C]putrescine incorporation decreased when ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid was added, while it increased with the addition of A23187.

Keywords putrescine incorporation; transferrin uptake; reticulocyte; endocytosis

Introduction

Polyamines such as putrescine, spermidine and spermine are widely distributed in mammalian cells.[1] The interaction of polyamines with the plasma membranes induces several effects, *e.g.* inhibition of the mobility of glycoproteins within the plane of the plasma membrane,[2] and inhibition of the association of protein kinase C with membrane.[3] Koenig *et al.*[4] reported that testosterone induced a rapid stimulation of endocytosis following sustained increase in polyamines in mouse kidney cortex. They provided the hypothesis that polyamines served as intracellular messengers to regulate transmembrane Ca^{2+} movements, Ca^{2+}-dependent membrane transport functions. However, the detailed role of polyamines in this endocytosis is not clear.

To investigate the relationship between polyamine and endocytosis, we studied the movement of extra-additional labeled putrescine during transferrin uptake by rat reticulocytes.

Materials and Methods

Isolation of Reticulocytes Reticulocytes were obtained from male Wistar rats weighing 200 to 250 g which were bled 3 d after receiving 3 successive daily injections of phenylhydrazine (Nakarai Tesque) at a dose of 40 mg/kg body weight. Reticulocyte-rich blood was washed three times with Eagles' minimum essential medium (MEM, Difco Laboratories) and reticulocytes were purified by centrifugation in density gradients of Percoll (Pharmasia) at $5000 \times g$ for 15 min.[5] About 90% of total erythroid cells was obtained as reticulocytes which were determined by staining with new methylene blue. Purified cells were suspended with the incubation buffer (150 mM NaCl, 50 mM Tris, 5 mM glucose, pH 7.4) at a density of 1×10^8 cells/ml.

Assay for Transferrin Uptake Rat transferrin (Sigma) was labeled with ^{125}I by the method of Markwell.[6]

Four hundred microliters of cell suspension was incubated with 100 μl of the incubation buffer containing a final concentration of 80 nM [^{125}I]transferrin (specific activity, 250 GBq/mmol), 1 μM $FeCl_3$, and 1 mg/ml bovine serum albumin for 1 h at 4 °C. After the incubation, unbound-ligands were removed, the cells were washed twice with the incubation buffer, and then incubated with 500 μl of the same buffer containing a final concentration of 1 mM $CaCl_2$ for various time periods at 37 °C. After the incubation, 1 ml of ice-cold stopping buffer (150 mM NaCl, 50 mM Tris, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol, 1 mg/ml bovine serum albumin) was added to the cell suspension in order to terminate transferrin uptake. Immediately, reticulocytes were centrifuged at $2000 \times g$ for 10 min and washed twice with the stopping buffer. The cells were then treated with 0.06% trypsin (Difco Laboratories) at 4 °C for 30 min.[7] At the end of incubation, trypsin inhibitor (from bovine pancreas, type I, Sigma) was added to give a final concentration of 0.4 mg/ml. The cells were precipitated by the centrifugation. The supernatant radioactivity was used as cell surface bound ligands and the cell radioactivity was used as internalized ligands. The radioactivity was determined by a NaI scintillation counter (Aloka, ARC-300). Non-specific binding of [^{125}I]transferrin was determined in the presence of 200-fold concentration of unlabeled transferrin and amounted to less than 6% of the total binding.

Assay for Putrescine Incorporation [^{14}C]Putrescine incorporation was determined as follows. After reticulocytes were prebound with 80 nM unlabeled transferrin, unbound ligands were removed. The cells were incubated with the incubation buffer containing a final concentration of 1 μM [1,4-^{14}C]putrescine dihydrochloride ([^{14}C]putrescine, specific activity, 2 GBq/mmol, New England Nuclear) and 1 mM $CaCl_2$ for various time periods at 37 °C. After the incubation, 1 ml of ice-cold stopping buffer containing a final concentration of 1 mM putrescine was added to the cell suspension in order to terminate transferrin uptake and putrescine incorporation. After reticulocytes were treated with trypsin as described above, the cells were exposed to hypo-osmotic buffer (NaCl-free stopping buffer) for 10 min at 37 °C to induce hemolysis. The protein was precipitated with 10% trichloroacetic acid (TCA) and then dissolved in 1 M NaOH. ^{14}C-Radioactivity was determined by a liquid scintillation counter (Beckman LS7800). Non-specific incorporation of [^{14}C]putrescine was determined in the presence of 200-fold unlabeled putrescine and amounted to less than 2% of the total incorporation.

Fractionations of Cell Membrane and Cytosol Distribution of incorporated [^{14}C]putrescine was determined as follows. After hemolyzing reticulocytes with hypo-osmotic stopping buffer as mentioned above, the lysate was centrifuged at $20000 \times g$ for 40 min. This process was repeated three times. The pellet thus obtained was used as the cellular membrane fraction. Supernatants were collected and then proteins were precipitated with 10% TCA and used as the cell cytosol fraction. Each fraction was dissolved in 1 M NaOH, and ^{14}C-radioactivity was determined.

Treatment of Reagents A23187 (Calbiochem) was dissolved in dimethylsulfoxide (DMSO) such that, upon addition to the cells, DMSO concentration would not exceed 0.5%. Ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) was dissolved in the incubation buffer.

Measurement of $^{45}Ca^{2+}$ Influx Reticulocytes were incubated with the incubation buffer containing 1 mM EDTA at 4 °C for 60 min in order to remove extracellular Ca^{2+}. Cells prebound with transferrin were then incubated in the incubation buffer containing 1 μM $^{45}Ca^{2+}$ (specific radioactivity 66.7 GBq/mmol, New England Nuclear) for the indicated time periods at 37 °C. After the incubation, 1 ml of ice-cold stopping buffer containing 1 mM $CaCl_2$ was added to the cell suspension. Immediately, reticulocytes were centrifuged and washed with the stopping buffer. The cells were dissolved in 1 M NaOH, and radioactivity of $^{45}Ca^{2+}$ was determined using the liquid scintillation counter. Non-specific $^{45}Ca^{2+}$ influx was determined in the presence of 1 mM $CaCl_2$ and amounted to less than 3% of total radioactivity.

Results and Discussion

Transferrin uptake by reticulocytes time-dependently increased at 37 °C (Fig. 1A) and reached a plateau after 10 min incubation. The amount of transferrin released from cell surface by trypsin treatment time-dependently decreased following incubation at 37 °C (Fig. 1B). This decrease was complementary to the increase in internalized transferrin. At 4 °C, however, little uptake of transferrin occurred even after 20 min (Fig. 1A, B). These results are in accord with those of the previous report of Kohno and

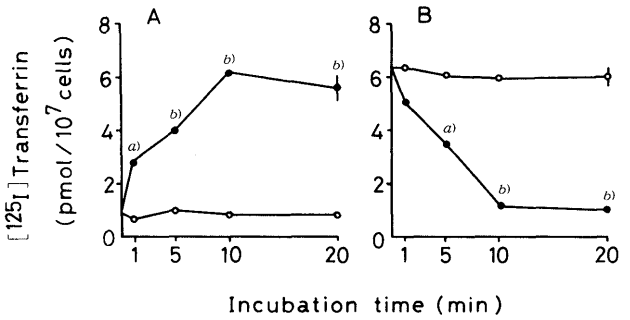

Fig. 1. Time Courses of Transferrin Uptake by Reticulocytes

Reticulocytes were incubated with 80 nM [^{125}I]transferrin for 1 h at 4 °C. After unbound ligands were removed, reticulocytes were further incubated at (●) 37 °C or (○) 4 °C for the indicated time periods. (A) Internalized or (B) cell surface bound ligands was determined as described in Materials and Methods. Each point is the mean ± S.D. of three samples. Significantly different from at 4 °C by Student's *t*-test (*a*) $p<0.01$, *b*) $p<0.001$).

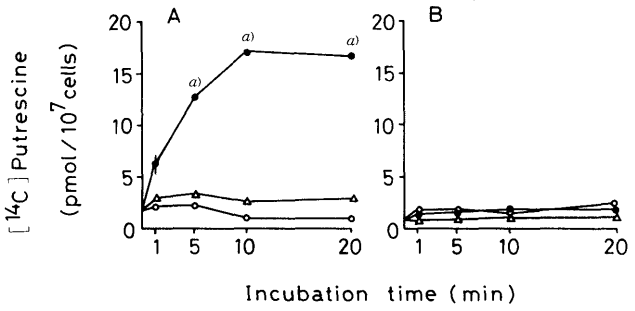

Fig. 2. Time Courses of Putrescine Incorporation into Reticulocytes during Transferrin Uptake

Reticulocytes were incubated (●, ○) with or (△) without 80 nM unlabeled transferrin for 1 h at 4 °C. After unbound ligands were removed, reticulocytes were further incubated in the presence of 1 μM [^{14}C]putrescine at (●, △) 37 °C or (○) 4 °C for the indicated time periods. (A) Internalized or (B) cell surface bound [^{14}C]putrescine was determined as described in Materials and Methods. Each point is the mean ± S.D. of three samples. Similar results were obtained in two separate experiments. Significantly different from at 4 °C or without transferrin by Student's *t*-test (*a*) $p<0.001$).

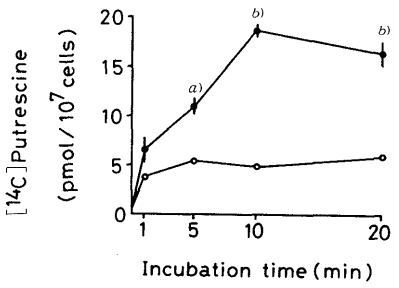

Fig. 3. Time Courses of Putrescine Incorporation into the Protein of Plasma Membrane or Cytosol in Reticulocytes during Transferrin Uptake

Reticulocytes were incubated with 80 nM unlabeled transferrin for 1 h at 4 °C. After unbound ligands were removed, reticulocytes were further incubated in the presence of 1 μM [^{14}C]putrescine at 37 °C for the indicated time periods. Fractionations of (●) membrane and (○) cytosol were performed as described in Materials and Methods. Each point is the mean ± S.D. of three samples. Similar results were obtained in two separate experiments. Significantly different from cytosol by Student's *t*-test (*a*) $p<0.01$, *b*) $p<0.001$).

Tokunaga.[7] To learn whether polyamine is involved in the process of transferrin uptake, the putrescine incorporation into the cells during this process was measured. When reticulocytes were incubated with [^{14}C]putrescine at 37 °C in the presence of transferrin, putrescine incorporation into reticulocytes, as well as the transferrin uptake process, was saturated for 10 min (Fig. 2A). However, no putrescine was incorporated by reticulocytes at 4 °C (Fig. 2A), nor did putrescine incorporation occur in the absence of transferrin (Fig. 2A). These results suggest that putrescine incorporation is dependent on transferrin uptake. To rule out the possibility that the incorporated putrescine is bound to transferrin, cell surface bound ligands were determined. No putrescine was released by trypsin treatment following incubation at 37 °C in the presence of transferrin (Fig. 2B). These results indicated that putrescine incorporation occurred during transferrin uptake without binding to the ligands. Reticulocytes were separated into cytosol and membrane fractions after incubation with transferrin at 37 °C to determine the distribution of incorporated putrescine into the cells. The time course of putrescine incorporation in each fraction is illustrated in Fig. 3. Incorporation into the protein of cytosol fraction did not change at all during the process of transferrin uptake, while time-dependent increases in putrescine incorporation into the protein of membrane fraction were found. This pattern closely resembled that by whole cells. These results indicate that putrescine incorporation is induced by transferrin and occurs through a different route from that of transferrin uptake.

It has been reported that the activation of cell surface receptors by hormones and other external stimuli causes a

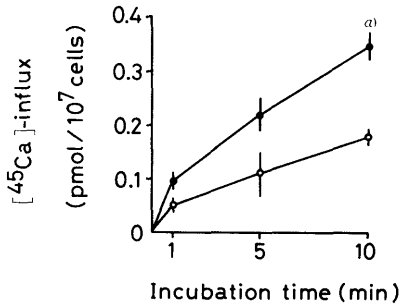

Fig. 4. ^{45}Ca-Influx into Reticulocytes during Transferrin Uptake

Reticulocytes were incubated (●) with or (○) without 80 nM unlabeled transferrin for 1 h at 4 °C. After unbound ligands were removed, reticulocytes were further incubated in the presence of 1 μM ^{45}Ca at 37 °C for the indicated time periods. Radioactivity of ^{45}Ca was measured as described in Materials and Methods. Each point is the mean ± S.D. of three samples. Similar results were obtained in two separate experiments. Significantly different from without transferrin by Student's *t*-test (*a*) $p<0.01$).

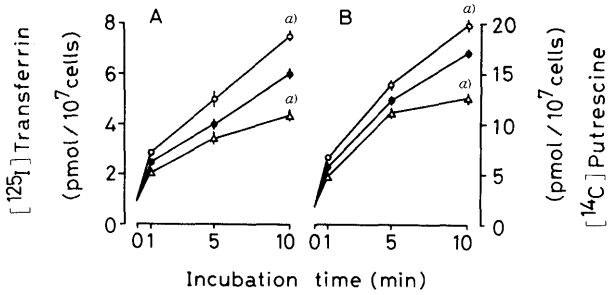

Fig. 5. Effect of A23187 or EGTA on Transferrin Uptake and Putrescine Incorporation into Reticulocytes

Reticulocytes were incubated with 80 nM (A) ^{125}I-labeled or (B) unlabeled transferrin for 1 h at 4 °C. After unbound ligands were removed, reticulocytes were further incubated (B) with or (A) without 1 μM ^{14}C-labeled putrescine at 37 °C for the indicated time periods in the presence of (○), 1 μM A23187 or (△) 1 mM EGTA or (●) vehicle. (A) Internalized [^{125}I]transferrin or (B) [^{14}C]putrescine was determined as described in Materials and Methods. Each point is the mean ± S.D. of three samples. Similar results were obtained in three separate experiments. Significantly different from control by Student's *t*-test (*a*) $p<0.05$).

rapid influx of Ca^{2+}.[8,9] Therefore, to investigate whether transferrin uptake is related to extracellular Ca^{2+} influx, reticulocytes were incubated with 1 μM $^{45}Ca^{2+}$ in the presence and absence of transferrin. As shown in Fig. 4, transferrin slightly induced Ca^{2+}-influx into the cells in a time-dependent manner. In exploring whether a relationship between putrescine incorporation and Ca^{2+} influx exists, we found that 1 mM EGTA slightly inhibited both transferrin uptake and putrescine incorporation (Fig. 5). This suggests that the process of transferrin uptake or putrescine incorporation requires extracellular Ca^{2+}. This suggestion is further supported by results of the effect of Ca^{2+}-ionophore on this process. A23187 slightly enhanced both putrescine incorporation and transferrin uptake (Fig. 5). From these findings, it is suggested that putrescine is incorporated into the plasma membrane protein by a Ca^{2+}-dependent mechanism.

Piacentini and Beninati[10] found that putrescine covalently linked to protein *via* a γ-glutamyl linkage in acid-insoluble fraction of freshly isolated hepatocytes. They suggested that transglutaminase, a Ca^{2+}-dependent enzyme, mediated post-translational modification through polyamine incorporation.[10,11] Maxfield *et al.*[12] proposed that transglutaminases might be involved in aggregation of receptors *via* cross-linking of the proteins in endocytosis. Furthermore, Dadabay and Pike[13] reported a rapid increase in transglutaminase activity of A431 cells following treatment with epidermal growth factor which was taken up by receptor-mediated endocytosis. From these findings, there is a possibility that polyamines are provided as the substrate for transglutaminase in the process of receptor-mediated endocytosis.

Further detailed studies are necessary to explain the putrescine incorporation in this paper and are now in progress.

References

1) C. Tabor and H. Tabor, *Annu. Rev. Biochem.*, **53**, 749 (1984).
2) M. Schindler, D. E. Koppel and M. P. Sheetz, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 1457 (1980).
3) R. J. H. Wojcikiewicz and J. N. Fain, *Biochem. J.*, **255**, 1015 (1988).
4) H. Koenig, A. Goldstone and C. Y. Lu, *Biochem. Biophys. Res. Commun.*, **106**, 346 (1982).
5) E. Kimura, T. Suzuki and Y. Kinoshita, *Nature* (London), **31**, 1201 (1960).
6) M. A. K. Markwell, *Anal. Biochem.*, **125**, 427 (1982).
7) H. Kohno and R. Tokunaga, *J. Biochem* (Tokyo), **97**, 1181 (1985).
8) A. D. Goldstone, H. Koenig, C. Y. Lu and J. J. Trout, *Biochem. Biophys. Res. Commun.*, **114**, 913 (1983).
9) G. N. Mozhayeva, A. P. Naumov and Y. A. Kuryshev, *Biochim. Biophys. Acta*, **1011**, 171 (1989).
10) M. Piacentini and S. Beninati, *Biochem. J.*, **249**, 813 (1988).
11) M. Piacentini, N. Martinet, S. Beninati and J. E. Folk, *J. Biol. Chem.*, **263**, 3790 (1988).
12) F. R. Maxfield, M. C. Willingham, P. J. A. Davies and I. Pastan, *Nature* (London), **277**, 661 (1979).
13) C. Y. Dadabay and L. J. Pike, *Biochemistry*, **26**, 6587 (1987).