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Effect of a Capsid-Stabilizing Pyridazinamine, R 78206, on the Eclipse and Intracellular Location of Poliovirus

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R 78206 (a pyridazinamine derivative) inhibits the formation of poliovirus eclipse particles. Its effect on the intracellular location of poliovirus was studied by separating subcellular fractions in iso-osmotic Nycodenz gradients. The compound did not inhibit internalization of intact virus into small lipid vesicles, but it did inhibit the release of virus from these vesicles and its entry into lysosomes.

Poliovirus has been postulated to undergo uncoating inside the cell (10). Consistent with this finding is the observation that the virions enter the cell by receptor-mediated endocytosis and probably undergo uncoating along the endocytotic pathway (17). According to a different view, poliovirus is modified at the plasma membrane and uncoating is initiated from the outside (6).

Various structurally unrelated, antiviral compounds have been used as an aid to study the early phases of picornavirus replication. Several WIN products (oxazolinyl isoxazole compounds synthesized by Sterling-Winthrop Research Institute, Rensselaer, N.Y.) and R 61837 (one of a series of pyridazinamine compounds synthesized by Janssen Research Foundation, Beerse, Belgium) have been shown by X-ray crystallography to bind into a hydrophobic pocket underneath the canyon of human rhinovirus 14 (1, 3, 14), thus stabilizing the viral capsid. Arildone (WIN 38020), disoxaril (WIN 51711), and R 78206 (a pyridazinamine derivative) prevent poliovirus replication. They also stabilize the viral capsid in vitro against thermal or alkali denaturation (2, 5, 11, 13).

Methods of subcellular fractionation were developed to study the intracellular locations of infecting virions and eclipse particles, using both rate zonal and isopycnic centrifugation in Nycodenz gradients (8, 9). Infecting virions and eclipse particles can be traced to four principal subcellular fractions defined by their positions in rate zonal Nycodenz gradients. The bottom region (BR) of the gradient contains plasma membrane fragments, the middle region (MR) contains lysosomes, and the top region (TR) contains cytosol and small lipid vesicles (TR vesicles). The viral particles that are free in the cytosol can be separated from those associated with TR vesicles by isopycnic centrifugation. The plasma membrane and the lysosomes have been defined after assaying for the presence of marker enzymes such as alkaline phosphatase and *N*-acetyl-β-D-glucosaminidase. The TR vesicles are detergent-sensitive structures, and the virus in these vesicles is inaccessible to poliovirus antibodies. These fractionation techniques have now been applied to study the effect of R 78206 on the intracellular movements of poliovirus.

Previous work in this laboratory has shown that intact virions that had been taken up at 26°C were modified to 135S and 80S particles upon incubation of the cells at 37°C (4, 9). At 26°C, virions were associated with the plasma membrane and TR vesicles. When the temperature was raised to 37°C, viral particles were found mainly in lysosomes and in the cytosol (8).

To examine the effect of R 78206 on intracellular poliovirus modification, [^{35}S]methionine-labeled Mahoney type 1 poliovirus was adsorbed at 26°C to HeLa cells at an input multiplicity of 6,000 virions per cell. After 2 h, cells were washed and a sample was taken. At that time, about 25% of input virus was found to be associated with the cells. The remaining cells were further incubated for 0.5 h at 37°C with or without R 78206. The cells were then examined in two ways. Detergent cell lysates were analyzed by sucrose gradient centrifugation to show the eclipse particles (Fig. 1A to D), and cell homogenates were analyzed by rate zonal Nycodenz gradients to ascertain the intracellular location of the viral particles (Fig. 1E to H). After virus uptake at 26°C, all radioactivity was associated with intact 160S virions (Fig. 1A), and these virions were located in both the BR and TR (Fig. 1E). Subsequent incubation of the cells at 37°C without inhibitor caused the formation of 80S and 135S eclipse particles (Fig. 1B) and the transfer of radioactivity to both MR and TR (Fig. 1F), confirming previous results (8). In the presence of 10 ng of R 78206 per ml, both the formation of eclipse particles (Fig. 1C) and the transfer to MR (Fig. 1G) were severely reduced; with 100 ng/ml, both were totally suppressed (Fig. 1D and H). It is concluded that R 78206 inhibited poliovirus eclipse as well as the entry of particles into the lysosomal MR and that the two effects showed the same dose dependence.

MR fractions were described as being lysosomal on the basis of the colocalization of the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase (8). To further confirm the inhibition of viral entry into lysosomes by R 78206, rate zonal Nycodenz fractions were assayed for the enzyme. As can be seen from Fig. 2A, the lysosomal enzyme profile cosedimented with MR. In the presence of 10 ng of R 78206 per ml, however, the lysosomes were still present, but viral entry was inhibited (Fig. 2B).

We subsequently investigated whether R 78206 interfered with the emptying of TR vesicles. HeLa cells were preincubated at 26°C with labeled virus, washed, and reincubated at 37°C either without or with 100 ng of R 78206 per ml. As in the foregoing experiment, the cells were examined in two ways. One portion was analyzed by sucrose gradient cen-

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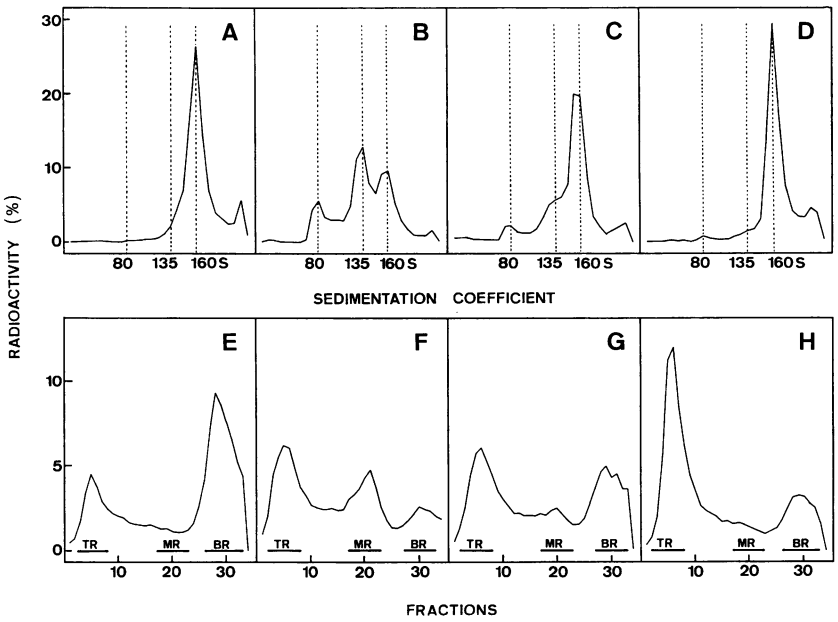

FIG. 1. Effect of R 78206 on the processing of virions into lysosomes. HeLa cells were preincubated for 2 h at 26°C with ^{35}S-labeled virus. After collection of a sample (A and E), the cells were washed and reincubated for 0.5 h at 37°C without R 78206 (B and F) or with the compound at the concentration of 10 (C and G) or 100 (D and H) ng/ml. One portion of the cells was detergent lysed by addition of Nonidet P-40 to a final concentration of 0.1%, and the postnuclear supernatant was analyzed in 15 to 30% sucrose gradients in phosphate-buffered saline (pH 8) (A to D). A second portion was resuspended in isotonic sucrose solution (8.5% [wt/vol] sucrose, 1 mM EDTA, 5 mM Tris-HCl [pH 8]) and mechanically homogenized by back-and-fourth pushes between two syringes with 21-gauge needles connected by 10 cm of polyethylene 0.76-mm-bore tubing. The postnuclear supernatants were then analyzed by rate zonal centrifugation in 2 to 30% Nycodenz gradients (E to H). Thirty percent Nycodenz was made in 5 mM Tris-HCl (pH 8) supplemented with 1 mM EDTA and diluted in isotonic sucrose to achieve lower concentrations (full details are provided in references 8 and 9). Solutions of R 78206 were made in dimethyl sulfoxide. In all mixtures, dimethyl sulfoxide was equalized to 0.001%. The 100% values were 3,791 (A), 1,843 (B), 1,892 (C), 3,645 (D), 18,947 (E), 9,375 (F), 9,753 (G), and 18,217 (H) cpm.

trifugation to reveal the eclipse particles. The results (not shown) were similar to those shown in Fig. 1. A second portion underwent rate zonal centrifugation on Nycodenz gradients. At 26°C, virus was associated mainly with the TR and BR (Fig. 3A). Again, the results showed the transfer of radioactivity to MR at 37°C (Fig. 3B) and the inhibition of viral accumulation in MR by R 78206 (Fig. 3C). To separate virus in TR vesicles from virus free in the cytosol, the pooled TR fractions were then recentrifuged to equilibrium. TR vesicles equilibrated around fraction 15, and cytosolic viral particles equilibrated in fractions 27 to 28 (Fig. 3D). As expected (8), at 37°C, all radioactivity had vanished from the TR vesicles (Fig. 3E), but this was not so in the R 78206-treated cells, in which TR vesicles still existed and contained viral particles (Fig. 3F). Thus, R 78206 appeared to inhibit the release of virus from TR vesicles.

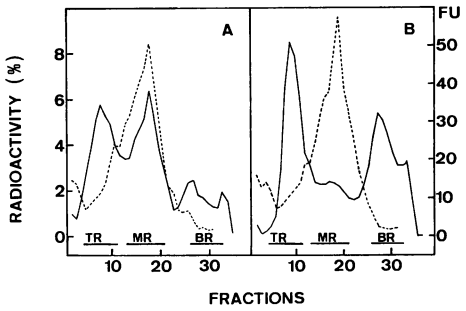

FIG. 2. Distribution of a lysosomal marker enzyme. HeLa cells were preincubated for 2 h at 26°C with ^{35}S-labeled virus. Cells were washed and further incubated for 0.5 h at 37°C without R 78206 (A) or with 10 ng of the compound per ml (B). Cells were then washed and homogenized without detergent. The homogenates were submitted to rate zonal Nycodenz gradient centrifugation. Of each gradient fraction, 0.3 ml was used for the radioactivity profile and 50 μl was used to assay for *N*-acetyl-β-D-glucosaminidase, using 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide dissolved in methoxyethanol as described previously (12). Bold lines, radioactivity profile; dashed lines, lysosomal enzyme profile as measured in fluorescence units (FU). The 100% values were 4,641 (A) and 9,412 (B) cpm.

In all experiments reported in this paper, the cells were preincubated at 26°C. However, it was verified that TR vesicles were also formed when the cells were directly infected at 37°C with R 78206; thus, the TR vesicles were not an artifact of preincubation at the lower temperature (results not shown).

To summarize, R 78206 inhibits the modification of virions to eclipse particles. The compound does not prevent binding to the plasma membrane (BR), but it appears to block the exit of virions from TR vesicles, and it blocks viral entry into lysosomes. These findings suggest a link between capsid modification to 135S particles and entry of viral particles into lysosomes. Our results may help to distinguish between two plausible sequences: either 160S virions are first converted to 135S particles, which are then transported to lysosomes, or conversely, 160S virions are first transported to lysosomes, there to be modified to 135S particles. Our observation that R 78206-stabilized virions did not accumulate in the

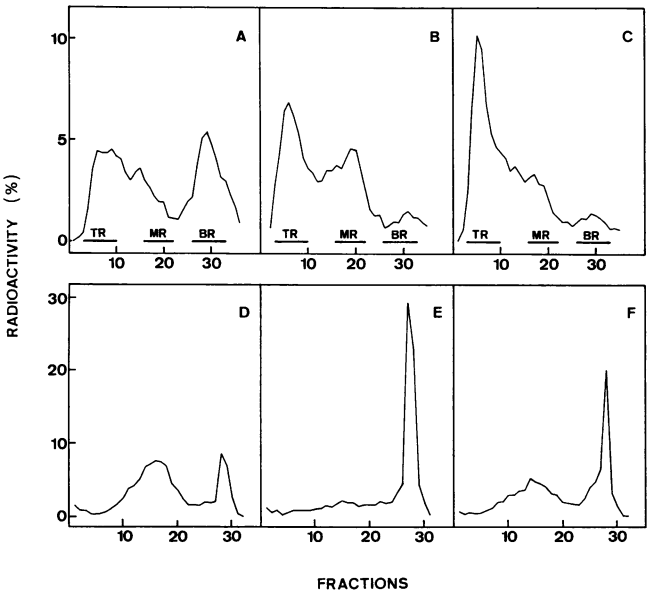

FIG. 3. Effect of R 78206 on the processing of virions from TR vesicles. HeLa cells were preincubated for 2 h at 26°C with ^{35}S-labeled virus. After collection of a sample (A and D), the cells were washed and reincubated for 0.5 h at 37°C without inhibitor (B and E) or with 100 ng of R 78206 (C and F) per ml. The collected cells were homogenized without detergent, and 0.1 ml of each 0.4-ml gradient fraction was submitted to rate zonal Nycodenz centrifugation (A to C). The remainder of the TR peak fractions (shown by the bar) was recentrifuged to equilibrium (D to F) in 10 to 30% Nycodenz gradients underlaid with a Maxidens cushion. The 100% values were 4,778 (A), 2,688 (B), 3,114 (C), 2,054 (D), 1,687 (E), and 2,591 (F) cpm.

lysosomes renders the latter possibility unlikely. This lack of accumulation of intact virus in lysosomes is rather unexpected, since it had been shown that stabilization of the viral capsid with disoxaril did not interfere with viral uptake through receptor-mediated endocytosis up to endosomes and/or lysosomes (16). The observation is probably not an artifact imputable to the use of chemicals to stabilize the virions, since a similar effect was indeed observed at 26°C, a temperature too low for virus modification (9) but above the 20°C threshold temperature for transport of different ligands to lysosomes (7).

Thus, when modification to 135S was inhibited either by chemicals or by low temperature, the 160S virions did not accumulate in lysosomes but instead remained at the plasma membrane and in TR vesicles, possibly still bound to their receptors. Different intracellular pathways of receptor-ligand complexes during endocytosis have been recognized (e.g., reference 15). In one pathway, both receptor and ligand are transported to the lysosomes. Our results may indicate that this is not the case for poliovirus.

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