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Uptake and Processing of Human Platelet Factor 4 by Hepatocytes (42626)

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Abstract. We previously demonstrated rapid clearance of human platelet factor 4 (PF4) from rabbit and rat blood, its accumulation in the liver, and elimination of PF4 degradation products in urine. The purpose of the present experiments was to characterize interaction of PF4 with cultured rat hepatocytes. ^{125}I-PF4 was taken up by hepatocytes reaching maximum at 180 min. The association of ^{125}I-PF4 with hepatocytes was two times greater at 37°C than at 4°C. At 37°C degradation of ^{125}I-PF4 by hepatocytes was also observed as indicated by the increase of ^{125}I-PF4 radioactivity soluble in 6% trichloroacetic acid. By contrast, no uptake of ^{125}I-β-thromboglobulin antigen was observed. Autoradiography demonstrated that short incubation (5–20 min) of ^{125}I-PF4 with hepatocytes results in the association of ^{125}I-radioactivity with cell membranes while after longer incubation (60 min) radioactivity was also localized in the endosomes. Heparin inhibited binding and uptake of ^{125}I-PF4 radioactivity by hepatocytes. We propose that part of PF4 released in the circulating blood by activated platelets is bound to the surface of hepatocytes and that it is further processed by these cells.

Platelet factor 4 (PF4) is a specific platelet α granule protein that is released by stimulated platelets by secretion (1). PF4 binds with high affinity to heparin and other glycosaminoglycans and neutralizes anticoagulant activity of heparin (2). It also has a number of activities that suggest an involvement in inflammation and tissue repair (3, 4). PF4 rapidly disappears from human (5), primate (6), rabbit (7), and rat (8) circulation following a biphasic exponential curve. It has been shown that PF4 binds *in vitro* to the endothelial cells (9), and it has been suggested that *in vivo* PF4 binds to sites on the endothelium from where it can be brought back to the circulation by heparin (5, 8). A recent report from our laboratory demonstrated accumulation and catabolism of PF4 in liver (7). The purpose of the present study was to investigate further the role of the liver in PF4 catabolism by characterizing the interaction of ^{125}I-PF4 with cultured rat hepatocytes. Radiolabeled β-thromboglobulin (βTG)antigen, a protein which shows 50% homology with PF4 (10) and which is known to be catabolized by kidney (7, 11) was used for comparative purposes.

Materials and Methods. *Isolation, purification and labeling of human platelet factor 4 and β-thromboglobulin antigen with ^{125}I-PF4.* This was done as described previously (7). The specific radioactivities of the labeled proteins were 20–50 μCi/μg (4×10^7–10^8 dpm/μg).

Distribution of ^{125}I-radioactivity in rat organs after injection of ^{125}I-labeled antigen. Five minutes after injection of 20 μCi of ^{125}I-labeled PF4 per kilogram body weight, the animals were killed by intravenous injection of sodium pentobarbital. The liver, lungs, heart and spleen were removed immediately and washed with 0.15 *M* NaCl. Total radioactivity was counted in each organ by slicing it into small pieces, counting all pieces, and summing the net counts.

Isolation and primary culture of rat hepatocytes. We followed the method of Moriarity and Savage (12) as modified by Shimoyama *et al.* (13). In brief, adult male albino rats weighing 200–300 g were anesthesized with intraperitoneal sodium pentobarbitol and the inferior vena cava was cannulated to provide a route for perfusing liver while the portal vein provided an outflow. In

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TABLE I. DISTRIBUTION OF ^{125}I-PF4 RADIOACTIVITY IN RAT ORGANS AFTER INJECTION OF PLATELET PROTEIN (MEAN ± SD)

Organ	% Total radioactivity recovered after 5 min
Liver	34.1 ± 17.4
Lungs	2.9 ± 1.7
Kidney	7.0 ± 1.8
Spleen	5.6 ± 4.5
Heart	0.8 ± 0.4

order to remove blood, liver was perfused with 200 ml of calcium-free Hanks' balanced salt solution containing 0.5% bovine serum albumin, 0.5 m*M* EDTA, 1 m*M* magnesium sulfate, 1.0 U/ml heparin, 100 μg/ml of streptomycin, 1000 U/ml penicillin, and 2 mg sodium bicarbonate (pH 7.35). The perfusion was then continued with 170 ml Hank's balanced salt solution in which EDTA and heparin were replaced by 2.5 m*M* calcium chloride and 0.05% (w/v) of collagenase (Sigma, St. Louis, MO). After 20 min of perfusion, the liver was transferred aseptically to a container containing 20 ml of collagenase solution. The hepatocytes were dispersed by cutting any chunks with fine sterile scissors and drawing up and down with a wide bore capillary pipette. The cell suspension was filtered through a coarse (153 μ^2) mesh and then through fine (64 μ^2) mesh nylon gauze. The cells were plated at about 2 × 4 10^4 cells/cm^2 in Earle's minimum essential medium (MEM) containing 15% newborn calf serum (Gibco, Grand Island, NY) and 20 m*M* Hepes and incubated for 24 hr on tissue culture dishes (60 × 15 cm) at 37°C in air:CO_2. One liver yielded 200–400 × 10^6 essentially pure parenchymal cells with 85% viability as determined by Trypan blue exclusion. As determined by phase microscopic examination, the hepatocyte cultures after 24 hr were essentially homogeneous; the cells were epithelioid, distinctly different from that of spread-out endothelial and Kupfer cells.

Interaction of ^{125}I-PF4 or ^{125}I-βTG antigen with hepatocytes. After 24 hr incubation of

TABLE II. UPTAKE OF HUMAN ^{125}I-PF4 AND ^{125}I-βTG ANTIGEN BY RAT HEPATOCYTES AND ITS ALTERATION BY HEPARIN

Platelet protein	Incubation time (min)	Temperature (°C)	Heparin	^{125}I radioactivity (cpm per pellet)
^{125}I-PF4	1	4	−	156 ± 6
	60	4	−	1170 ± 176*
	1	4	+	108 ± 1
	60	4	+	138 ± 16
	1	37	−	249 ± 70
	60	37	−	2102 ± 229*
	1	37	+	132 ± 20
	60	37	+	216 ± 35
^{125}I-BTG	1	4	−	67 ± 8
	60	4	−	90 ± 46
	1	4	+	68 ± 21
	60	4	+	111 ± 48
	1	37	−	62 ± 2
	60	37	−	112 ± 5
	1	37	+	65 ± 1
	60	37	+	138 ± 43

Note. Hepatocytes were prepared from rat liver as described under Materials and Methods. ^{125}I-PF4 or ^{125}I-βTG (60,000 cpm or 12 ng per dish containing 2 × 10^5 cells) was added alone or in a combination with 5 μg heparin prepared from porcine intestine (Elkins Sinn, Inc., Cherry Hill, NJ) and incubated for 1 or 60 min at 4 or 37°C. After incubation, the supernate fluid was removed, and cells were washed three times in the same medium and harvested in 0.15 *M* NaCl by means of a rubber policeman. The pellets were centrifuged and ^{125}I radioactivities were counted. The values are means and SD from three experiments. The protein content in each pellet was 350 μg, approximately.

* Statistically significant; difference, $P \leq 0.02$.

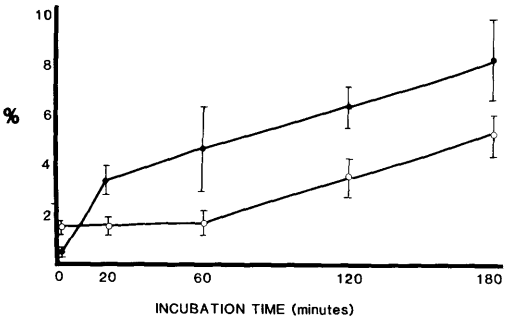

FIG. 1. Uptake and degradation of ^{25}I-PF4 by hepatocytes. The hepatocytes were prepared as described under Materials and Methods and in Table I, (100,000 cpm, or 15 ng) ^{125}I-PF4 was added per dish. The figure represents percentage of total ^{125}I radioactivity (●) recovered in hepatocyte pellet after various incubation times at 37°C and percentage of total ^{125}I radioactivity not precipitated by 6% trichloroacetic acid from conditioned medium (○). The data are presented as mean values and standard deviations from six experiments. The difference in the amount of ^{125}I radioactivity soluble in TCA after 1 min incubation and after 120 (or 180 min) incubation is significant at $P \leq 0.002$ level.

the plated hepatocytes, the medium was removed and the cells (2×10^5 per dish) were washed three times with an excess of serum-free MEM. ^{125}I-PF4 or ^{125}I-βTG was added alone or in combination with 5 μg heparin (prepared from porcine intestine, Elkins Sinn, Inc., Cherry Hill, NJ) and incubated for various time intervals at 4 or 37°C. After incubation, the supernatant fluid was removed, and cells were washed three times in the medium and harvested in 0.15 *M* NaCl by means of a rubber policeman. The pellets were centrifuged and ^{125}I radioactivity was counted. In parallel experiments the supernates from cultures were taken at various time intervals and treated with 6% trichloroacetic acid (TCA). The radioactivity in the supernates and in the TCA precipitates was counted.

Autoradiography. For electron microscope autoradiography, pale gold or silver sections (50–100 nm) were picked up on copper grids coated with 0.3% celloidin and affixed to glass slides with double-sided Scotch tape. Under a light brown Ilford safelight, slightly gelled Ilford L-4 emulsion (diluted 1:1) was applied to the grids by the loop method of Caro *et al.* (14). The emulsion-coated sections were placed in light-tight boxes, sealed, and exposed at 4°C for appropriate periods. After exposure, the photographic emulsion was developed for 5 min in freshly made, filtered Microdol-X, and the grids were washed briefly in distilled water, fixed in Kodak photographic fixer, washed, and air dried. The autoradiographs were stained with uranyl acetate followed by lead citrate. Samples were examined and 7-11 cell profiles micrographed at each time point. The number of silver grains associated with the membrane and endosomes was determined by probability circles (15).

Results. We found in four experiments that human ^{125}I-PF4 injected intravenously to rats accumulated predominantly in liver. Five minutes after injection of ^{125}I-PF4 the percentage of the total radioactivity recovered in rat liver amounted to 34.1 ± 17.4% (mean ± SD). The remaining radioactivity was present in lungs, kidney, spleen, and heart (Table I). At that time significant PF4 radioactivity was still present in blood. Similar values were obtained previously following injection of radiolabeled PF_4 into rabbits (7).

Table II shows association of human ^{125}I-PF4 with rat hepatocytes following a 60-min incubation period. This association was about two times greater at 37°C than at 4°C. It was completely inhibited by heparin. By contrast, there was no association of ^{125}I-βTG with hepatocytes under the same conditions.

It can be seen from Fig. 1 that uptake of ^{125}I-PF4 was time dependent since it did not reach saturation in 120–180 min incubation at 37°C. At this period of time we observed degradation of ^{125}I-PF4 at 37°C as indicated by an increase in the conditioned medium of

TABLE III. DISTRIBUTION OF SILVER GRAINS

Time of incubation (min)	Number of grains/cell profile	
	Membrane	Endosome associated
0	0	0
5	6.1	1.5
20	6.7	1.7
60	12.0	7.8

^{125}I-radioactivity soluble in 6% trichloroacetic acid. The degradation of ^{125}I-PF4 did not occur following incubation of hepatocytes with ^{125}I-PF4 at 4°C or following incubation of hepatocytes with ^{125}I-PF4 at 37°C in the presence of 5 μg heparin per dish. A possible interpretation of the data presented in Fig. 1 is that binding was complete within the period to the first measurement (20 min), and that there was additional processing through the first 60 min and then a steady state was achieved in which uptake paralleled degradation. Preliminary experiments suggested that uptake of ^{125}I-PF4 by hepatocytes at 37°C was dose dependent at the concentration range of PF4 of 50 to 20,000 ng per dish (2 $\times$ 10^5 cells). The uptake of PF4 was not saturable under these experimental conditions (data not shown).

Electron microscopic autoradiography showed that binding and internalization of PF4 increased with time, with a sharp increase between 20 and 60 min (Table III). Internalization at 60 min had increased much more (5.2-fold over 5 min) than binding (2.0-fold). The process by which hepatocytes internalized PF-4 is shown in Figs. 2–4.

Discussion. In our previous study we demonstrated that human platelet factor 4 accumulated in the liver and that it was catabolized in this organ (7). The experiments presented in this paper show that hepatocytes do indeed catabolize PF4 and that uptake of PF4 by hepatocytes is specific. However, endothelial and Kupfer cells may also catabolize PF4.

The following similarities between PF4 catabolism in liver studied by means of nu-

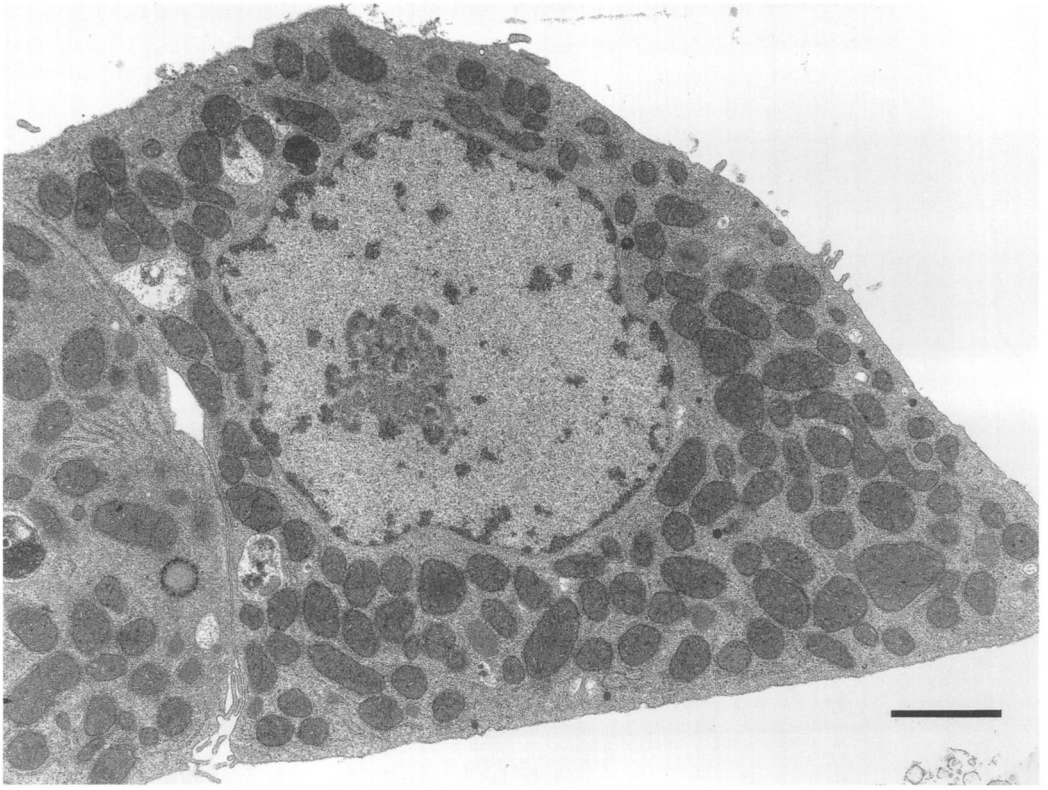

FIG. 2. Rat hepatocyte fixed 24 hr after isolation. This culture was not exposed to PF4 and was not covered with photographic emulsion. Cells appeared essentially healthy with characteristic organelles including nucleus, numerous mitochondria, and rough endoplasmic retciulum. Cells had developed pseudopodia and cell junctions had formed between cells as had bile canaliculae. Bar equals 2 μM.

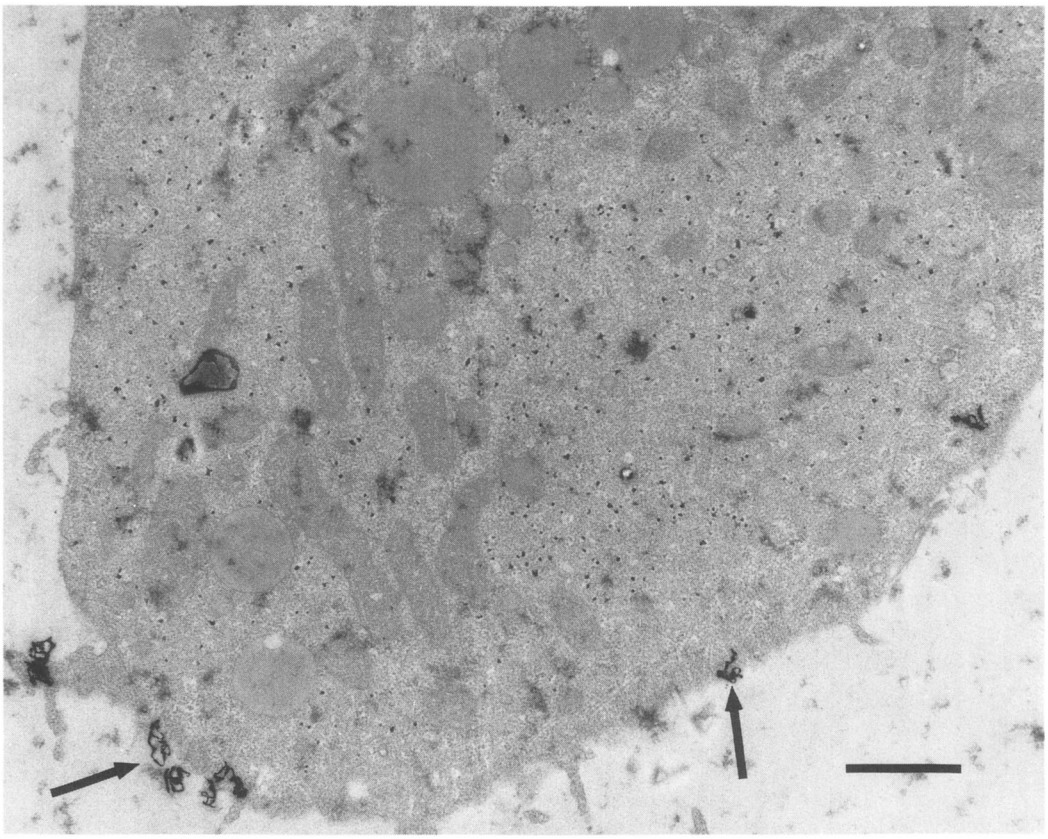

FIG. 3. Profile of one cell from companion culture incubated with ^{125}I-labeled PF4 for 20 min at 37°C before being prepared for autoradiography. Characteristic silver grains (arrows) were located predominantly on the cell surface or immediately internal to the membrane. Bar equals 1 μM.

clear imaging technique (7) and that in cultured hepatocytes were observed. The rate of uptake and degradation of ^{125}I-PF4 by isolated hepatocytes (Fig. 1) was of the same order as uptake and disappearance of ^{131}I-PF4 radioactivity from the rabbit liver (7). The uptake of βTG antigen by liver *in vivo* was minimal and its catabolic rate in this organ was several times lower than that of PF4 (7). In the system of cultured hepatocytes no uptake of ^{125}I-βTG was observed. Moreover, nuclear imaging studies demonstrated that heparin prevented accumulation of PF4 in liver (7), whereas the present experiments showed inhibitory effect of heparin on uptake of ^{125}I-PF4 by hepatocytes (Table II). The specificity of PF4 processing by hepatocytes is suggested by the observation that these cells do not take up βTG antigen, a platelet protein which is structurally related to PF4 (10).

The observations made with electron microscope autoradiography indicated that hepatocytes bound and processed PF4 through receptor-mediated endocytosis; i.e., the labeled ligand was bound to the cell surface and then moved into the cell interior where it appeared in small vesicles, large translucent vesicles, and multivessicular bodies (Figs. 3, 4). Taken together with the observation that acid-soluble label appeared in the supernate after prolonged incubation, it may be suggested that hepatocytes processed PF4 by classical receptor-mediated endocytosis (16). Further study will be required to overcome limitations imposed by the low solubility of

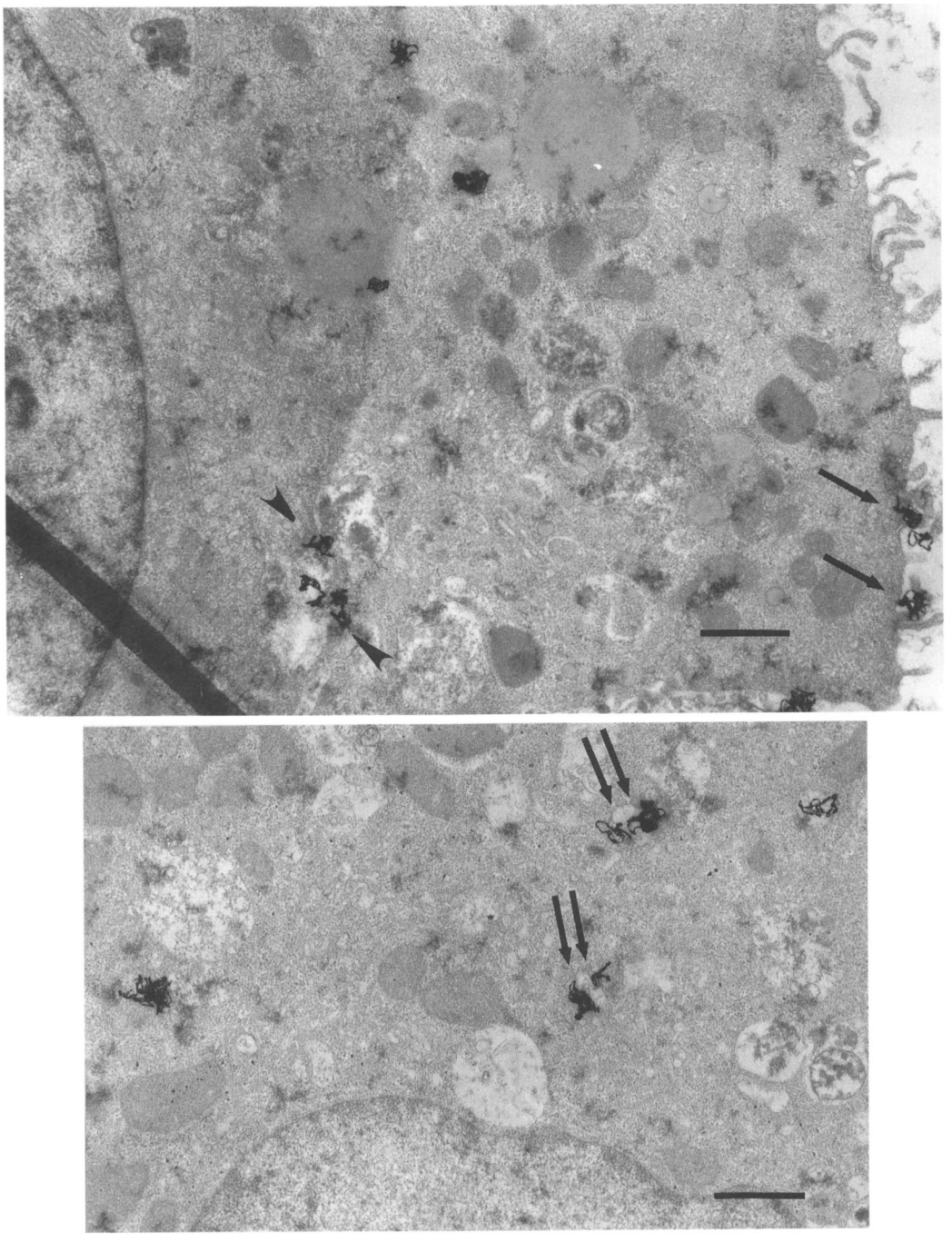

FIG. 4. Profiles of two cells from companion culture incubation with ^{125}I-labeled PF4 for 60 min before being prepared for autoradiography. Silver grains (arrows) were still located on the surface but many were well within the cell. Given the limited resolution of autoradiography, it appeared that some silver grains were associated with uncoated, translucent endosomes (double arrows). Others were closely associated with multivesicular endosomes that contained some amorphous material (arrowheads). The number of endosomes of various sizes and configurations were greatly increased over that of control hepatocytes or those incubated with PF4 for 20 min. Bar equals 1 μM.

PF4 and to characterize the process with respect to kinetics and to the fate of receptors. Shifman and Pizzo (17) demonstrated receptor-mediated catabolic clearance of thrombin–antithrombin III complex by rat liver hepatocytes. It is known that PF4 neutralizes completely the anticoagulant activity of endothelial cell heparin or heparin sulfate glycosaminoglycans and that it delays formation of thrombin–antithrombin III complex (18, 19). A possible role of platelet factor 4 in the clearance of thrombin–antithrombin III complexes awaits further studies.

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