THE EFFECT OF Ca^{2+} AND TRIFLUOPERAZINE ON THE PROCESSING OF HUMAN ACETYLATED LOW DENSITY LIPOPROTEIN BY NON-PARENCHYMAL LIVER CELLS

Theo J. C. VAN BERKEL, Jan F. NAGELKERKE and Johan K. KRUIJT
Department of Biochemistry I, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands

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1. Introduction

Studies with human fibroblasts have defined a receptor-mediated pathway by which cells take up and degrade low density lipoprotein (LDL), the major cholesterol(ester)-transport protein in human plasma [1]. Internalization of the particle is followed after recognition by a specific receptor. These receptors are clustered in coated regions of the cell membrane, so-called coated pits. Upon LDL binding the coated pits invaginate and form coated endocytotic vesicles which fuse subsequently with lysosomes. The protein moiety and the cholesterol esters are then degraded by the action of cathepsins and acid cholesterol esterase, respectively. Although the different steps involved in receptor-mediated endocytosis have been described, the molecular mechanisms involved in the process are largely unknown. Peritoneal macrophages possess a binding site for acetylated LDL which is distinct from the native LDL receptor [2]. On macrophages this binding site is coupled to a very active internalization and degradation process. This property facilitates the study of the molecular mechanism of the uptake and degradation pathway. This is also illustrated by the finding that after injection of ^{125}I-labeled acetyl-LDL into rats, these particles are rapidly cleared from the circulation (<3 min). The radioactivity is subsequently merely recovered in the non-parenchymal liver cells (unpublished). This study describes the in vitro binding of both native and acetylated LDL to freshly isolated non-parenchymal liver cells. Binding of acetylated LDL to these cells is only twice as high as the binding of native LDL but the degradation is increased 30–50-fold after acetylation of the LDL. The degradation of acetylated LDL is inhibited by chloroquine and NH_4Cl, indicating a lysosomal process. Mg-EDTA at 2 mM inhibits the degradation of acetyl-LDL by 50% and trifluoperazine (50 μM), an inhibitor of calmodulin [3,4], blocks the degradation completely. The rate of association of acetyl-LDL with non-parenchymal cells is only slightly inhibited by trifluoperazine. It is concluded that the main action of trifluoperazine is exerted on the route of acetyl-LDL to the lysosomes after the initial binding process. The data are consistent with a role of calmodulin in the receptor-mediated endocytotic process although it cannot be excluded that trifluoperazine interacts with another still unknown target.

2. Materials and methods

2.1. *Isolation, modification and labeling of LDL*

Human LDL was isolated exactly as in [5]. The density range was 1.019 -1.063. LDL was acetylated with repeated additions of acetic anhydride as in [6]. In short, 1 ml LDL (at 2–10 mg protein/ml) in 0.15 M NaCl, 1 mM EDTA and 8 mM phosphate buffer (pH 7.5) was added to 1 ml of a saturated solution of sodium acetate with continuous stirring in an ice-water bath. Next, acetic anhydride was added in multiple small aliquots (2 μl) over 1 h. After the addition of a total mass of acetic anhydride equal to 1.5-times the mass of protein used, the mixture was stirred for an additional 30 min. The solution was then dialyzed overnight at 6°C against buffer containing 0.15 M NaCl, 1 mM EDTA and 8 mM phosphate buffer (pH 7.5). Both LDL and acetyl-LDL were iodinated at pH 10 by the ICl method [7], as modified for lipoproteins [8]. Free I^- was removed by Sephadex G-50 filtration. The iodine:protein ratio was between 0.6–0.8 atoms/mol for both LDL and acetyl-LDL. Of the radioactivity in the preparation, 3–4% was free, 3–5% was present in phospholipids

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and 92–94% was protein-bound. The only apoprotein present in LDL is apolipoprotein B as determined on SDS–polyacrylamide electrophoresis [10].

2.2. *Isolation of liver cells*

Isolation of rat non-parenchymal liver cells was performed by differential centrifugation (method 2 in [9]).

2.3. *Lipoprotein binding, uptake and degradation*

Incubations of freshly isolated liver cells with the indicated amounts of lipoproteins and cells were performed in Ham's F-10 medium (modified), containing 5% (v/v) human lipoprotein-deficient serum (LPDS; final protein 2.5 mg/ml). The incubations were carried out either in plastic Eppendorf tubes in 1 ml total vol. or in 25 ml Erlenmeyer flasks (siliconized) stoppered with rubber caps with a total incubation volume and time as indicated in the figure legends. At the indicated times 1 ml samples were withdrawn and the cells were centrifuged in an Eppendorf centrifuge for 2 min at 3000 rev./min. The pellets were suspended in 1 ml medium containing 50 mM Tris–HCl (pH 7.4), 0.15 M NaCl and 2 mg bovine serum albumin, incubated for 5 min at 4°C and centrifuged again. This washing procedure was repeated twice. The last washing was performed with 0.15 M NaCl only to enable a reliable protein determination. The cell-associated radioactivity and 0.5 ml of the different supernatants were counted in a LKB-Wallace ultrogamma counter. The radioactivity in the last supernatant was <5% of the cell-associated radioactivity. Degradation of the lipoproteins was measured as in [11]. To 0.5 ml of the first supernatant, 0.2 ml 35% trichloroacetic acid was added followed by incubation for 15 min at 37°C; subsequently the mixture was centrifuged for 2 min at 15 000 rev./min. To 0.5 ml of the supernatants 5 μl 40% KI and 25 μl 30% H_2O_2 were added. After 5 min at room temperature 0.8 ml $CHCl_3$ was added and the mixture was shaken for another 5 min. After centrifugation for 2 min at 15 000 rev./min, 0.4 ml of the aqueous phase (containing iodinated amino acids and small peptides) and 0.5 ml of the $CHCl_3$ phase (containing I_2 formed from I^- by oxidation with H_2O_2) was counted. This sample was corrected for quenching by $CHCl_3$. In the corresponding blanks the lipoproteins were incubated in the absence of cells. Further additions are indicated in the figure legends.

The viability of the cells was >95%. The viability was also checked after addition of chloroquine or trifluoperazine. It was found that high chloroquine concentrations (>100 μM) lead to a lower viability of the cells especial at the longer incubation times. With the concentrations used in this paper no decrease in cell viability was observed.

2.4. *Reagents*

Collagenase (type I) was obtained from Sigma. Trifluoperazine was supplied by Smith, Kline and French Labs.

3. Results

Fig.1 shows the time course of the interaction of ^{125}I-labeled LDL and ^{125}I-labeled acetylated LDL with the freshly isolated non-parenchymal cells. The amount of cell-associated lipoprotein rapidly increases and levels off at the longer incubation times. With rat lipoproteins, the amount of cell-associated radioactivity remained constant from 0.5–3 h incubation [10]. Such a steady-state level is not readily observed with human LDL or acetyl-LDL, although in some experiments the increase in cell-associated radioactivity from 2–3 h incubation was found to be low. The appearance of acid-soluble radioactivity in the water phase follows a completely different time dependency than the amount of cell-associated radioactivity. With acetyl-LDL a lag phase is evident (10–30 min) before the degradation reaches a constant rate (from 1–3 h). This degradation is completely blocked by chloroquine (100 μM, fig.2) or NH_4Cl (10 mM, not shown). Both unrelated compounds inhibit the lysosomal pathway of protein degradation [12]. So it can be concluded that acetyl-LDL follows an endocytotic route which involves the lysosomal compartment. Fig.2 also indicates that a similar inhibition of acetyl-LDL degradation occurs if 100 μM trifluoperazine is present. With 25 μM trifluoperazine intermediate inhibition is obtained. Fig.2 indicates that trifluoperazine also influences the initial phase of the interaction of acetyl-LDL with the cells. At shorter incubation times a lower amount of radioactivity is cell-associated than in the control incubation. However, this is not observed at prolonged incubation times, probably because the initial slight interaction of trifluoperazine with the binding of acetyl-LDL is compensated by the accumulation of acetyl-LDL due to an impaired degradation.

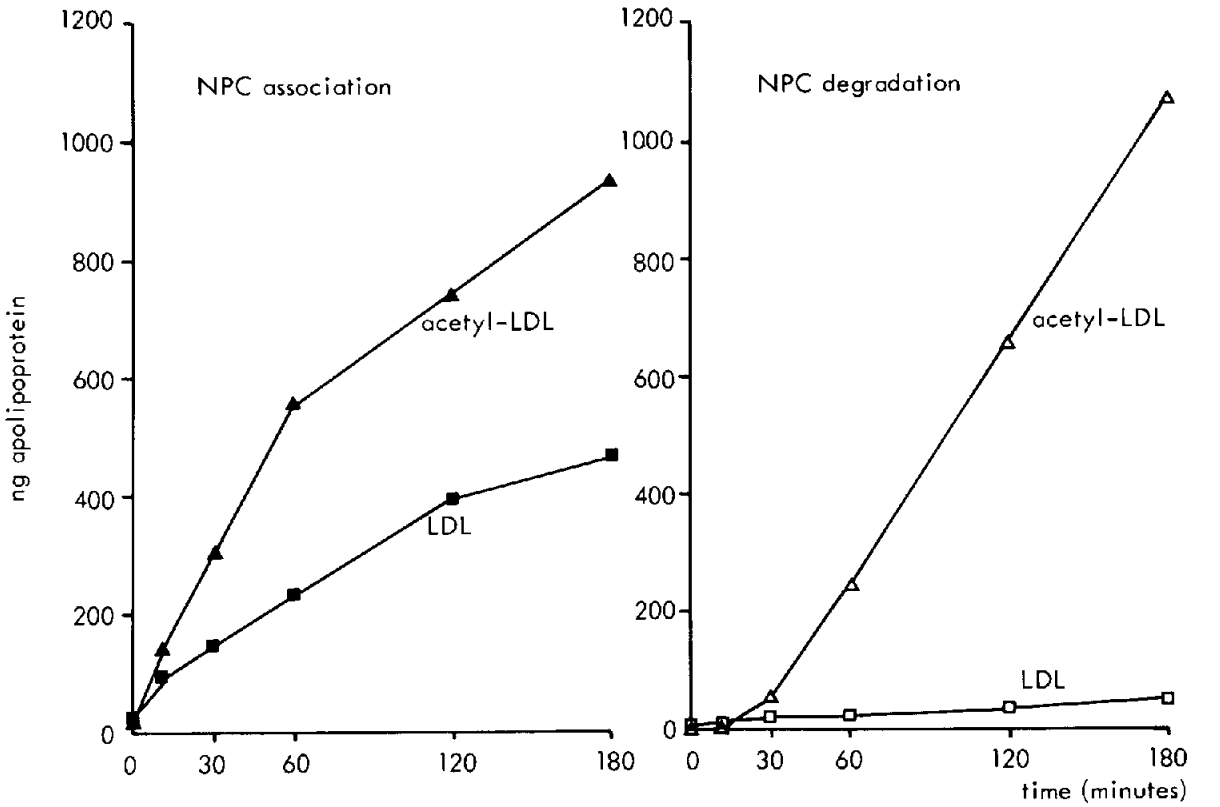


Fig.1. Time course of the cell association and degradation of LDL and acetyl-LDL by isolated non-parenchymal liver cells. The cells were incubated with 10.0 μg LDL/ml (■,□) or 10.1 μg acetyl-LDL/ml (▲,△). At the indicated time samples were withdrawn and the amount of cell-associated radioactivity (after 3 washings, left) as well as the radioactivity present in the acid-soluble water phase were determined (right). Both cell association and degradation are expressed as ng apolipoprotein/mg cell protein.

Trifluoperazine has been considered as a specific inhibitor of calmodulin, a protein which mediates the action of Ca^{2+} upon a number of cellular processes [4,13]. In [14] coated vesicles of brain were 7-fold enriched with calmodulin. The presence of calmodulin in the coated vesicles should be rationalized by a function in the endocytotic process. Fig.3 shows that the degradation of acetyl-LDL by non-parenchymal cells is hardly influenced by changes in the extracellular $[Ca^{2+}]$. In the presence of 2 mM Mg-EDTA, however, the degradation is ~50% of that measured in the absence of Mg-EDTA. From fig.3 it is also clear that this effect is not the consequence of the involvement of Ca^{2+} in the acetyl-LDL binding process, but apparently occurs at a step between binding and degradation. Fig.4 shows that the effective inhibitory concentration of trifluoperazine upon acetyl-LDL degradation is low. At 50 μM, trifluoperazine exhibits

Table 1
Effect of trifluoperazine upon the in vitro degradation of human iodine-labeled acetylated low density apolipoprotein by non-parenchymal cell homogenates

	Degradation (ng apolipoprotein . h^{-1} . mg cell $protein^{-1}$)
Acetyl-LDL	1832 ± 57
+ 100 μM trifluoperazine	1983 ± 50

The cells were homogenized by sonificating twice for 30 s at 21 kHz in a MSE ultrasonic desintegrator at 0°C. Incubations were carried out for 60 min at 37°C in acetate buffer (pH 4.2), 4 mM dithiothreitol and 10.8 μg/ml ^{125}I-labeled acetylated-LDL. The reaction was stopped by addition of 10% trichloroacetic acid and the acid-soluble water phase was obtained as indicated in section 2. For further details of the in vitro degradation measurements see [15]. The values are the mean ± SEM for 3 determinations

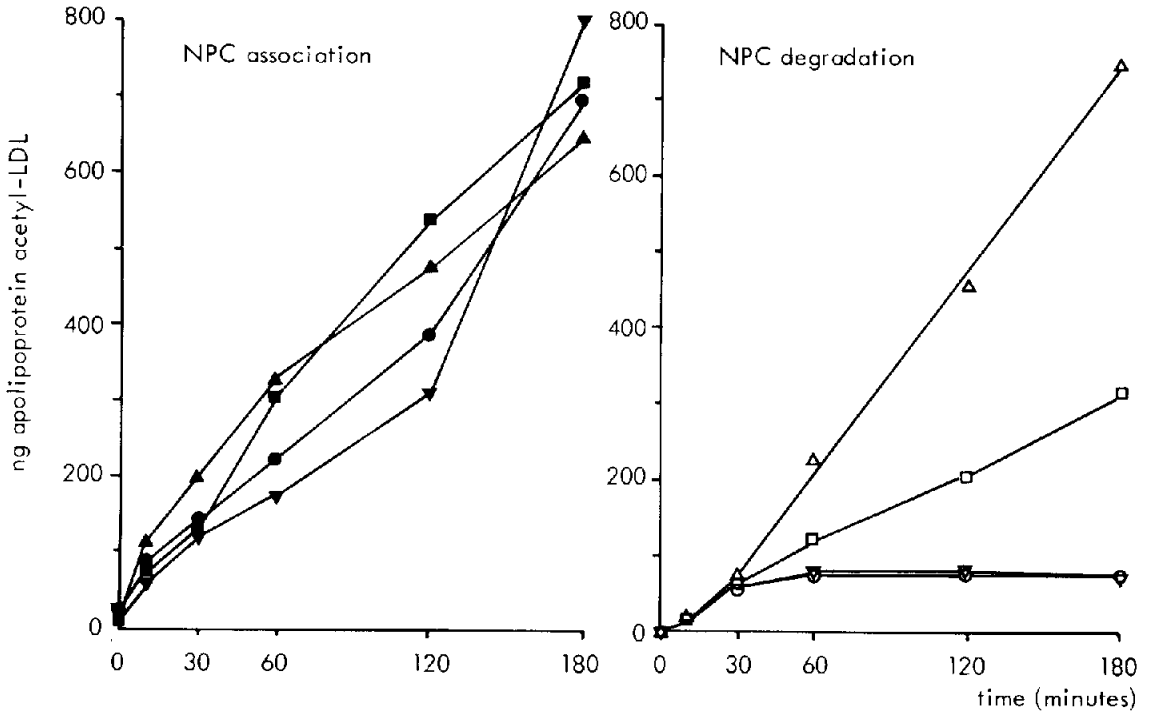
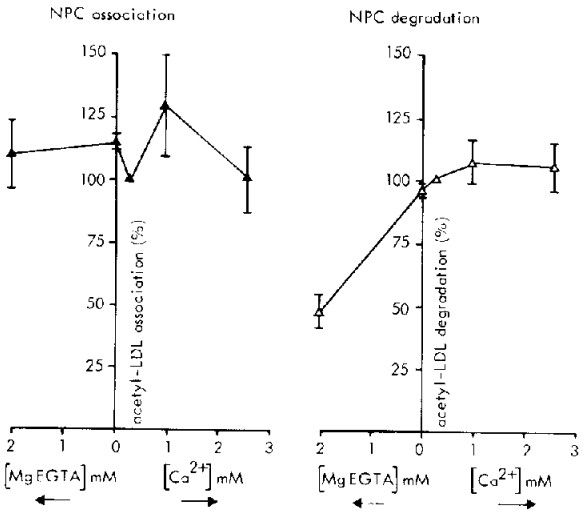


Fig.2. Effect of chloroquine and trifluoperazine upon the time course of the cell association and degradation of acetyl-LDL. The cells were incubated with 10.2 μg acetyl-LDL/ml in the absence (▲,△) or presence of: 100 μM chloroquine (▼,▽); 100 μM trifluoperazine (●,○); or 25 μM trifluoperazine (■,□). Both cell association (left) and degradation (right) are expressed as ng apolipoprotein/mg cell protein.

already a nearly complete inhibition and the half-maximal inhibitory concentration is ~20 μM. To verify whether trifluoperazine acts directly upon lysosomal enzymes we studied the effect of 100 μM trifluoperazine upon the acetyl-LDL degradation in a cell-free system at an optimal degradation pH of 4.2 (table 1). No inhibitory effect of trifluoperazine on the degradation by non-parenchymal cell homogenates was noticed.



4. Discussion

Non-parenchymal liver cells possess high affinity sites which can bind rat LDL and HDL [16]. The

Fig.3. The effect of Mg-EDTA and Ca^{2+} upon the cell-association and degradation of acetyl-LDL by isolated non-parenchymal cells. The cells were incubated 2 h with 5.5 μg acetyl-LDL/ml with the different indicated [Mg-EDTA] or [Ca^{2+}]. For the cells incubated with Mg-EDTA the last 2 washings before incubation, were also performed in the presence of 2 mM Mg-EDTA. The results were obtained with 3 different acetyl-LDL and cell preparations and are given as % of the association or degradation at 0.3 mM Ca^{2+} ± SEM. The 100% value for the cell association is 470 ng acetyl-LDL/mg cell protein and for the degradation 732 ng acetyl-LDL/mg cell protein.

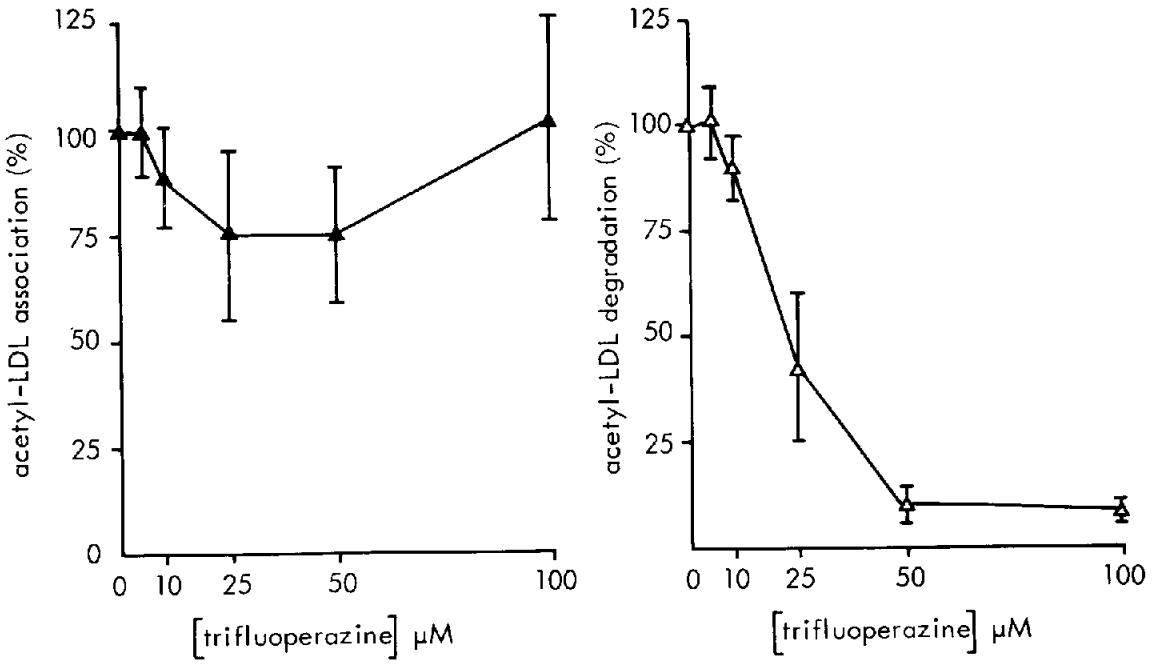


Fig.4. The effect of increasing trifluoperazine concentrations upon the cell association and degradation of acetyl-LDL by isolated non-parenchymal liver cells. The cells were incubated for 2 h with 10.3 μg acetyl-LDL/ml in the presence of the indicated amount of trifluoperazine. The results were obtained with 3 different acetyl-LDL and cell preparations and are given as % of the association or degradation in the absence of trifluoperazine ± SEM. The 100% value for the cell association is 690 ng acetyl-LDL/mg cell protein and for the degradation 844 ng acetyl-LDL/mg cell protein.

binding of rat LDL, HDL and also VLDL-remnants occurred at the same receptor. This receptor recognizes all these rat lipoproteins with a highest apparent affinity for VLDL-remnants [10]. Here we show that non-parenchymal cells bind and metabolize, in addition to the rat lipoproteins, both human LDL and acetyl-LDL. Although the cell-association of acetyl-LDL is only twice as high as for native LDL, the acetylation of native LDL increases the degradation 30–50-times. This high increase in degradation can be expected from macrophage-like cell types [2]. The degradation of acetyl-LDL by freshly isolated non-parenchymal liver cells is completely blocked by chloroquine (fig.2) or NH_4Cl (not shown). The incomplete inhibition during the first hour of incubation can be explained by the fact that the cells were not preincubated with these lysosomotropic agents. Together with the clear lag phase before degradation starts, this is strong evidence that the handling of acetyl-LDL follows the endocytotic route for receptor-mediated uptake, i.e., binding to a high affinity receptor (properties of this receptor will be reported elsewhere), uptake in endocytotic vesicles and degradation inside the lysosomes. These data show further that trifluoperazine, an inhibitor of calmodulin [34], inhibits the degradation of acetyl-LDL. The acetyl-LDL degradation is highly sensitive to trifluoperazine, with a half-maximal inhibition at 20 μM. Similar concentrations are reported to inhibit other calmodulin-mediated cellular processes [17,18]. Although this inhibition is consistent with a role of calmodulin in the endocytotic process the reported finding cannot be considered as conclusive. In [19] it was reported that the inhibitory action of trifluoperazine upon the effect of α-adrenergic agonists is due to a blockade of the binding of the α-adrenergic agonists to their receptor which is not mediated by calmodulin. For this reason, besides its action upon the degradation, we also investigated the effect of trifluoperazine upon the rate of cell-association of acetyl-LDL. It is shown that trifluoperazine indeed interacts with the initial binding (as does chloroquine), however, this does not explain the complete inhibition of the degradation of acetyl-LDL by trifluoperazine. Although a small inhibition of the cell-association occurs (at shorter incubation time), this inhibition is not observed at

prolonged incubation time probably because it is compensated by the main action of trifluoperazine, i.e., the blockade of the route to the lysosomes of acetyl-LDL, after the initial binding process. The action of trifluoperazine is probably not at the lysosomes themselves because the in vitro degradation of acetyl-LDL by cell homogenates at an acid pH is not inhibited by trifluoperazine. Further evidence that calmodulin is involved in the intracellular handling process of acetyl-LDL can also be deducted from the experiments in which the effect of Mg-EDTA and Ca^{2+} was tested. In contrast with most other ligand–receptor interactions [13,20], the association of acetyl-LDL with the cells was not Ca^{2+} or Mg-EDTA-dependent. This makes it possible to separate the cell-association process from the subsequent cellular handling and the data indicate that, in the presence of Mg-EDTA the degradation of acetyl-LDL is inhibited by ~50%. A complete inhibition was, however, not observed probably because extracellular Mg-EDTA is not able to deplete intracellular Ca^{2+} completely. In conclusion it can be stated that, in addition to the data in [14] where calmodulin was shown to be highly enriched in endocytotic vesicles, our data can be considered as evidence that calmodulin, or another still unknown target of trifluoperazine, is involved in the receptor-mediated endocytotic process.

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