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Effects of pimozide and penfluridol on the binding and endocytosis of α_2-macroglobulin-CH_3NH_2 by mouse peritoneal macrophages*

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α_2-Macroglobulin (α_2M) is a plasma glycoprotein which is unique in its ability to react and inhibit proteases from each of the four major classes [1]. Reaction of proteases with α_2M results in a conformational change in the inhibitor, detected electrophoretically as a "slow" to "fast" alteration in mobility [1–3]. This same conformational change occurs when α_2M reacts with methylamine which incorporates into the reactive site thiol-ester bonds of the α_2M molecule [1–5]. Conformational change exposes receptor recognition sites on α_2M, resulting in binding and endocytosis of "fast" form α_2M by macrophages [6, 7]. This process requires Ca^{2+} and is inhibited by EDTA [6, 7]. Inhibition of uptake in the absence of Ca^{2+} may be due to prevention of binding of "fast" form α_2M to the cell surface receptor. However, phagocytosis by rat peritoneal macrophages is accompanied by hyperpolarization of the cells, possibly by changing the membrane permeability to K^+ or Ca^{2+} [8–10]. In addition, the binding of asialo-orosomucoid to the Ashwell receptor on hepatocytes decreases both the influx and efflux of Ca^{2+} from the cell [11].

Trifluoperazine, a calmodulin inhibitor, inhibits capping of lectin on neutrophil leukocytes [12]. Another calmodulin inhibitor, chlorpromazine, inhibits clustering and endocytosis of α_2M on Swiss 3T3 cells [13]. However, since these drugs also have a variety of other toxic effects on the cell [14] besides inhibition of Ca^{2+}–calmodulin interaction [15–17], these results do not prove that Ca^{2+}–calmodulin interactions are necessary for binding and endocytosis of α_2M. Pimozide and penfluridol are highly specific Ca^{2+}–calmodulin inhibitors which possess an I_{50} about 10-fold lower than trifluoperazine or chlorpromazine [15–18]. We, therefore, investigated the possibility that a Ca^{2+}–calmodulin interaction is a part of the mechanism of the endocytosis of α_2M-CH_3NH_2 by examining the effects of pimozide and penfluridol on the binding and endocytosis of α_2M-CH_3NH_2 by mouse peritoneal macrophages.

Methods

Reagents. Pimozide and penfluridol, gifts of Dr. Michael Conn, were dissolved in dimethyl sulfoxide (DMSO) prior to use. Trypan blue was obtained from Sigma. Sheep red blood cells were the gift of Dr. Ralph Snyderman. Rabbit anti-sheep red blood cell IgC was from Kappel. $Na_2{}^{57}CrO_4$ was from New England Nuclear.

Cell binding studies. The preparation of mouse peritoneal macrophage monolayers and α_2M-CH_3NH_2, the labeling of α_2M-CH_3NH_2 with ^{125}I [19], and the binding experiments were performed as described in a previous publication [6]. In each experiment, a 100-fold molar excess of unlabeled α_2M-CH_3NH_2 was employed to determine nonspecific binding. For endocytosis experiments, macrophage monolayers were washed three times with Earle's balanced salt solution with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 10 mg/ml bovine serum albumin, pH 7.3, either with Ca^{2+} and Mg^{2+} (binding buffer A) or without Ca^{2+} and Mg^{2+} and with 5 mM EDTA (binding buffer B), and equilibrated at 37° for 1–2 hr. Reaction was begun by addition of radiolabeled ligand in a solution of binding buffer A or B and terminated by aspiration of the ligand solution and three washes with 1.0 ml binding buffer A or B at 4°. The pellets were washed three times with Earle's balanced salt solution with 25 mM Hepes, pH 7.3, at 4°, and the pellets were solubilized, counted for γ-radioactivity, and measured for protein content by the method of Lowry *et al.* [20] as modified by Peterson [21].

Macrophage function studies. Trypan blue exclusion studies were performed by incubating cell monolayers with various drug concentrations in Neumann–Tytell serumless medium at 37° for 2 hr. The medium containing the drug was aspirated, and the cells were washed three times with 1.0 ml of Hanks' balanced salt solution. A solution of 2.5 mg/ml trypan blue in Hanks' balanced salt solution was placed on the cell monolayers for 5 min at 25°. The trypan blue solution was aspirated, and the cells were washed three times with 1.0 ml of Hanks' balanced salt solution. Monolayers were examined by phase contrast microscopy.

Sheep red blood cell phagocytosis was determined as described by Koren *et al.* [22]. IgG coated sheep red blood cells were suspended at 1×10^9 cells/ml in Hanks' buffered salt solution and incubated for 1 hr at 37° with 10 μCi/ml $Na_2{}^{57}CrO_4$. Cells were washed three times and resuspended in Hanks' balanced salt solution. The ^{57}Cr-labeled cell suspension (0.5 ml) was incubated with the monolayers of 2.5×10^5 macrophages for 1 hr at 37°. The monolayers were washed once with Hanks' buffered salt solution, incubated with 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4, for 2 min at room temperature, and then washed two more times with Hanks' buffered salt solution. Monolayers were solubilized in 0.5% sodium dodecyl sulfate (SDS) and counted for γ-radioactivity. Sheep red blood cells which were not opsonized served as a control.

Results and discussion

The effects of differing Ca^{2+} concentrations on the uptake and degradation of [^{125}I]α_2M-CH_3NH_2 are summarized in Table 1. Maximal uptake and degradation occurred at about 1 mM Ca^{2+}. The small amount of uptake and degradation in the presence of 5 mM EDTA was due to nonspecific pinocytosis and degradation.

The toxicity of pimozide and penfluridol was determined by trypan blue exclusion studies. Figure 1 shows the percentage of cells excluding trypan blue following a 2-hr incubation with various concentrations of the inhibitors. The concentration of inhibitor at which only 50% of the cells excluded trypan blue (LC_{50}) was 12 μM for pimozide and 5 μM for penfluridol.

Figure 1 also shows inhibition of erythrocyte phagocytosis under the same conditions. Results are expressed as the percentage of the control containing DMSO but no inhibitor. At concentrations of inhibitor lower than the LC_{50} that was determined in the trypan blue exclusion studies, erythrophagocytosis was inhibited about 65% by penfluridol and 35% by pimozide.

Macrophage monolayers were incubated with 5 nM [^{125}I]-α_2M-CH_3NH_2 and either pimozide or penfluridol at 4° for 6 hr after a 2 hr, 37° incubation with various concentrations of inhibitors. Binding of α_2M-CH_3NH_2 to macrophages was unaffected by either pimozide or penfluridol. The uptake of 36 nM α_2M-CH_3NH_2 at 37° for 1 hr was determined after incubation of the cell monolayers at 37° for 2 hr with various inhibitor concentrations. Uptake of α_2M-CH_3NH_2 was also unaffected by the presence of either pimozide or penfluridol (Table 2).

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Table 1. Ca^{2+} dependence of uptake and degradation of [^{125}I]α_2M-CH_3NH_2 by macrophages*

Ca^{2+} (mM)	Uptake (pmoles/mg cell protein)	Degradation (pmoles/mg cell protein/min)
2.0	1.25 ± 0.12†	1.67
1.0	1.21 ± 0.06	1.54
0.5	1.04 ± 0.07	1.36
5 mM EDTA	0.09 ± 0.02	0

* Macrophages were incubated with 36 nM [^{125}I]α_2M-CH_3NH_2 for various times. Cell pellets were washed, harvested and subjected to γ-counting to determine uptake. The supernatant fraction from the incubation was cooled, made 10% in trichloroacetic acid (TCA), and centrifuged. The resulting supernatant fraction was subjected to γ-counting to determine TCA-soluble material at various times. Rate of degradation was determined from a least squares fit line of a plot of TCA-soluble material versus time; correlation coefficient > 0.9.

† Average ± S.D.

Previous studies by Dickson *et al.* [13] indicate that chlorpromazine inhibits clustering and endocytosis of α_2M by Swiss 3T3 cells. Since one effect of chlorpromazine is to inhibit Ca^{2+}–calmodulin interactions, we investigated the effect of two other inhibitors of Ca^{2+}–calmodulin interactions on the endocytosis of α_2M-CH_3NH_2 by macrophages. At concentrations of pimozide and penfluridol at which 90% of the cells excluded trypan blue, eythrophagocytosis, a process mediated by the Fc receptor on macrophage [22, 23], was inhibited 30% by pimozide and 60% by penfluridol. However, the endocytosis of α_2M-CH_3NH_2 was not affected under these conditions. Thus, phagocytosis by way of the Fc receptor has a different mechanism than the receptor-mediated endocytosis of α_2M-CH_3NH_2. Even though endocytosis requires Ca^{2+} and is accompanied by changes in membrane permeability to Ca^{2+} [11], these effects are independent of Ca^{2+}–calmodulin interactions.

Both of the inhibitors were toxic to macrophages; pimozide had an LC_{50} of 12 μM and penfluridol of 5 μM. There are numerous reports in the literature implicating Ca^{2+}–calmodulin interactions as a mediator of various cell functions on the basis of incubations of cells with high concentrations (50 μM) of these inhibitors (see Ref. 14 for review). Such results should be interpreted with caution, especially when experiments showing the viability of cells under these conditions are not reported.

In summary, we examined the effects of the calmodulin inhibitors pimozide and penfluridol on binding and endocytosis of α_2M-CH_3NH_2 by mouse peritoneal macrophages. The LC_{50}, as determined by trypan blue exclusion, was 12 μM for pimozide and 5 μM for penfluridol. The phago-

Table 2. Effects of pimozide and penfluridol on uptake and binding of α_2M-CH_3NH_2 by macrophages*

Drug (μM)	Specific binding (fmoles/mg cell protein) PM	PFD	Uptake (pmoles/mg cell protein) PM	PFD
0	131 ± 10	149 ± 10	1.7 ± 0.2	2.2 ± 0.3
0.1	143 ± 4	157 ± 5	1.5 ± 0.3	2.5 ± 0.3
0.5	154 ± 2	153 ± 5	1.8 ± 0.3	2.0 ± 0.2
1	159 ± 10	162 ± 10	2.0 ± 0.2	2.5 ± 0.2
2	ND†	138 ± 2	ND	2.3 ± 0.2
3	ND	126 ± 8	ND	2.0 ± 0.2
5	131 ± 2	ND	1.7 ± 0.2	ND
10	126 ± 5	ND	1.9 ± 0.3	ND

* Macrophage monolayers were incubated with the indicated concentration of pimozide (PM) or penfluridol (PFD) for 2 hr at 37°. For binding studies, the monolayers were cooled to 4° and incubated with the indicated concentration of inhibitor and 5 nM [^{125}I]α_2M-CH_3NH_2. Nonspecific binding was determined in the presence of 5 mM EDTA. The values shown are the mean ± standard deviation of three determinations. For uptake studies, the monolayers were incubated with 50 nM [^{125}I]α_2M-CH_3NH_2 and the indicated concentration of inhibitor for 1 hr at 37°. The results shown are the mean ± standard deviation of four determinations.

† Not determined.

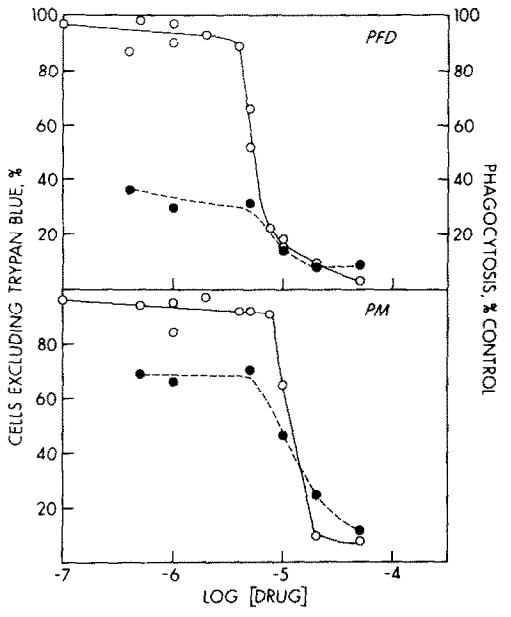

Fig. 1. Effects of pimozide and penfluridol on trypan blue exclusion and erythrophagocytosis by macrophages. Macrophage monolayers were incubated for 2 hr at 37° with various concentrations of penfluridol (PFD) or pimozide (PM). Key: (○) percentage of cells excluding trypan blue; and (●) erythrophagocytosis expressed as a percentage of control. Control is defined as monolayers incubated in the presence of DMSO but without PM or PFD.

cytosis of IgG coated sheep red blood cells was inhibited30% by 1 μM pimozide and 70% by 1 μM penfluridol, but 90% of the cells excluded trypan blue at these concentrations. The binding and endocytosis of α_2M-CH_3NH_2 were unaffected by 10 μM pimozide or 3 μM penfluridol.

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The effect of soman poisoning on phosphorylating capability and adenylate cyclase activity of isolated synaptosomal membranes

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In the preceding works Stitcher *et al.* [1] and Sevaljević *et al.* [2] have reported a significant increase of plasma cAMP in rats poisoned with soman (pinacolyl methylphosphonofluoridate) and discussed the possibility that it arose from an activation of adenylate cyclase via an ACh-induced release of humoral and pharmacologically active substances. This suggested that soman might affect protein phosphorylation which is a process involved in the regulation of various nervous tissue functions, particularly those related to synaptic transmission [3]. Along this line we have examined the effect of soman poisoning on protein kinase and adenylate cyclase activities in isolated synaptosomal membranes.

Male albino rats weighing 300–350 g were poisoned by a subcutaneous administration of 0.75 or 1.3 LD_{50} of soman (corresponding to 0.3–0.5 μmol/kg, respectively) and decapitated at the onset of convulsions. The cerebellum and medula oblongata were removed and the remaining tissue