Volume 281, number 1,2, 188–190 FEBS 09587 April 1991

ADONIS 001457939100297C

Stabilization of clathrin coated vesicles by amantadine, tromantadine and other hydrophobic amines

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Received 7 February 1991

Amantadine and related compounds stabilized the structure of purified pig brain clathrin coated vesicles (CCV) at biologically relevant concentrations. Incubation of purified CCV for 30 min at 25°C or 37°C caused the release of clathrin, as determined by a centrifugation assay, and a reduction in the number of coated vesicles, by electron microscopy. Amantadine (10 mM), tromantadine (1 mM), amidine D295 (cyclohexylcarboximidamide-(*N*-benzyl)hydrochloride) (10 mM), chloroquin (0.1 mM) and monodansylcadaverine (10 mM) significantly reduced the extent of dissociation.

Clathrin; Clathrin coated vesicle; Amantadine; Tromantadine; Hydrophobic amine

1. INTRODUCTION

Amantadine (1-aminoadamantane) is an FDA licensed anti-influenza A drug [1] and a treatment for Parkinson's disease [2]. Tromantadine (*N*-1-adamantyl-*N*-[2-(dimethylamino)ethoxy]acetamide-hydrochloride) is an amantadine derivative which inhibits early and late steps in herpes simplex virus replication including penetration [3], HSV induced syncytia formation and HSV glycoprotein processing [4]. Amidine D295 (cyclohexylcarboximidamide-(*N*-benzyl)hydrochloride) has similar anti-HSV activities but is less potent than tromantadine (unpublished data).

Amantadine and other lipophilic amines can inhibit infection by influenza A [1] as well as vesicular stomatitis virus, Semliki Forest and other viruses [5]. Their antiviral activity and ability to inhibit endocytic uptake of ligands have been attributed to their acidophilic/lysosomotropic properties [1,6]. Many lipophilic amines, including amantadine and its derivative, tromantadine, can also interact with membranes to stabilize their structure and prevent fusion processes [7,8].

Some hydrophobic amines and di- or polybasic amines interact with, and affect the function of clathrin and clathrin coated vesicles. CCV stabilization [9] and clathrin polymerization [10,11] can be promoted by di- or polybasic amines, e.g. spermine [9,11], hydrophobic aromatic amines, e.g. phenothiazines, chloroquin and quinine [10], or compounds with both structures, e.g. monodansylcadaverine [11]. Monodansylcadaverine can also inhibit internalization of certain ligand-receptor complexes [12] and the clustering of receptor bearing ligands on the cell surface [13].

In this study we examined the effects of amantadine, tromantadine and a tromantadine analogue, amidine D295 on clathrin-coated vesicles and compared their activity to the previously studied compounds, chloroquin and monodansylcadaverine. We identified another activity for amantadine and tromantadine which could contribute to their ability to inhibit ligand uptake.

2. RESULTS AND DISCUSSION

2.1. *Isolation of coated vesicles*

CCV were isolated from pig brains and separated by ultracentrifugation on sucrose density gradients according to Keen et al. [14]. The purified coated vesicles were collected and stored at 4°C in the final 5% sucrose fraction of the preparation. Samples were not frozen and were used within one week of isolation to minimize loss of integrity of the coated vesicles. All operations were performed at 4°C using MES buffer (0.1 M MES [2-(*N*-morpholino)ethane sulfonic acid] pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$ and 0.02% sodium azide, unless noted.

The CCV fraction and samples were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis on reducing 7.5% gels [15] and by electron microscopy. The predominant polypeptide in CCV fractions was clathrin with a molecular mass of 180000 Da but other CCV proteins at 100000, 50000, 36000 and 30000 Da were also detected. Intact CCV were

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Published by Elsevier Science Publishers B.V.

visualized by electron microscopy of uranyl acetate-negative-stained samples in a JEOL 100S JEM microscope. The CCV were identified by their characteristic size (55–70 nm), staining pattern and polygonal exostructure [16]. A small amount of uncoated vesicles and membranous debris was also visible in the samples.

2.2. *Dissociation of CCV*

Aliquots of clathrin-coated vesicles containing 45–60 μg/ml protein were pelletted at 130000 × *g* for 1 h and resuspended gently in either MES buffer, pH 6.5, different concentrations of the test compound in MES buffer or 0.5 M Tris-HCl at pH 7.0 and then incubated for 30 min at 25°C or 37°C. Studies were performed at pH 6.5 to maximize CCV stability and minimize CCV aggregation [9,11,17,18].

The extent of dissociation of CCV was determined by evaluation of the relative clathrin concentrations of supernatant and pellet samples (130000 × *g* for 30 min), as determined by densitometric scan of the Coomassie blue-stained band corresponding to clathrin on the SDS-PAGE. The densitometric readings were linear for protein concentration within the clathrin concentration ranges used in this study. Representative samples following each treatment were examined by electron microscopy to evaluate the coated vesicle samples for dissociation and clumping.

Whereas no clathrin release to the supernatant was observed after storage for as long as 3 days at 4°C, a 30-min incubation at 25°C caused release of 28% (SEM (standard error of sample mean) 2.4, $n = 3$) of the clathrin to the supernatant. Incubation of the CCV sample for 30 min at 37°C promoted dissociation of 41% (SEM 1.2, $n = 4$) of the clathrin from the CCV. Maximal release of clathrin from the CCV could be obtained by incubation of CCV at 37.5°C in 0.5 M Tris-HCl pH 7.0 at 37.5°C [14]. The Tris treatment caused release of 89.6% (SEM 1.5, $n = 4$) of the clathrin from CCV. The lack of basket-like structures and intact clathrin coats on vesicles by electron microscopy corroborated the disruption of the CCV.

2.3. *Treatment of CCV with tromantadine, amantadine, chloroquin, amidine 295, or monodansylcadaverine*

CCV were incubated with 0, 0.1, 1.0 or 10 mM amantadine (Sigma, St. Louis, MO), tromantadine (Merz, Frankfurt), amidine D295 (Merz, Frankfurt), chloroquin (Sigma, St. Louis, MO) or monodansylcadaverine (Sigma, St. Louis, MO) in MES buffer at pH 6.5 for 30 min at 37°C.

Incubation of the CCV suspension in 10 mM amantadine, amidine 295 or monodansylcadaverine; 1.0 mM tromantadine or 0.1 mM chloroquin significantly (5% level of significance as substantiated by Tukey Test statistical analysis [19]) reduced the release of clathrin into the supernatant fraction (Table I) (Fig. 1). The effects of the treatments on CCV were corroborated by qualitative electron microscopic observation. A higher proportion of intact, coated vesicles, as compared to control and Tris-treated samples, was observed following incubation with the effective concentrations of drug. Although both the CCV and polymerized clathrin structures would be expected to precipitate, CCV were the predominant structure seen in electron micrographs. Clumping, which would increase non-specific pelleting of CCV and confound the result, was not observed. The greater CCV-stabilizing activity of chloroquin corresponds with its reported activity on clathrin polymerization and stabilization [10]. The ability of amantadine, tromantadine and amidine D295 to stabilize CCV adds to the list of structural determinants which provide this activity.

The decrease in the levels of soluble clathrin following treatment of clathrin-coated vesicles with these

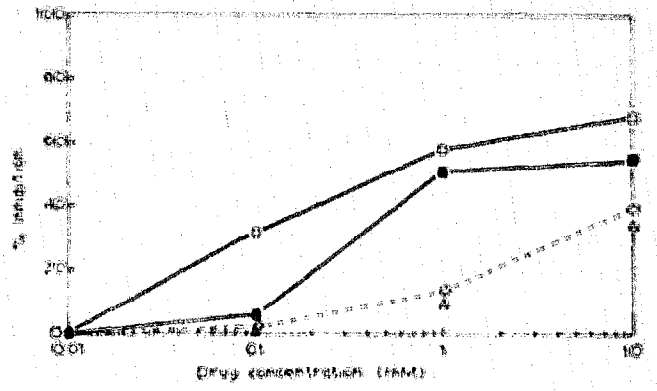

Fig. 1. Inhibition of clathrin-coated vesicle dissociation. Values were calculated using the formula below from the same data as for Table I following incubation with chloroquin (□), tromantadine (■), amidine D295 (○) and amantadine (△). [[%Pellet clathrin (treated) − %Pellet clathrin (control)]/%Supernatant clathrin (control)] × 100

Table I

Inhibition of clathrin release from clathrin-coated vesicles.

	Percent clathrin in pellet[1]			
Drug concentration	0	0.1 mM	1.0 mM	10 mM
Amantadine	58	58	62	72[2]
Amidine D295	59	59	64	75[2]
Tromantadine	59	61	80[2]	81[2]
Chloroquin	58	71[2]	82[2]	87[2]
Monodansylcadaverine[3]	39	36	47	77[2]

[1] Purified CCV were incubated in MES buffer or with different concentrations of drug for 30 min at 37°C, centrifuged and the supernatant and pellet analyzed by SDS-PAGE. The relative clathrin concentrations were determined from densitometric traces of the 180 kDa polypeptide band. The SEM (standard error of sample mean) was less than 2 for all samples.

[2] Significantly different from control as determined by Tukey test.

[3] Experiment performed independent of others.

compounds could result from stabilization of the CCV structures or enhanced repolymerization of clathrin into baskets. Stabilization of CCV could result from drug interactions with the clathrin cage or the membrane structure of the vesicle. Both amantadine and tromantadine will stabilize the bilayer phase of model phospholipid membranes [7]. As in this study, tromantadine was more effective than amantadine at stabilizing membrane structure.

Stabilization of CCV or aggregation of clathrin in vivo might limit the production of coated pits and new CCV and the fusion of CCV with other membranes. This could inhibit uptake of ligands, including influenza A, Semliki Forest and vesicular stomatitis virus by receptor mediated endocytosis [20], and impede other forms of intracellular traffic involving clathrin coated vesicles [21].

Acknowledgements: The study was supported in part by funds from Merz and Co., Frankfurt, Germany. We thank Jeannette Killius for assistance with the electron microscopy.

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