THE INHIBITORY EFFECT OF METHYLENEDISALICYLIC ACID ON THE ATTACHMENT OF RIBOSOMES TO MICROSOMAL MEMBRANES IN VITRO

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1. Introduction

The ribosome–membrane junction of the rough endoplasmic reticulum of eukaryotic cells is now recognized to play a central role in the processing and transmembrane transport of secretory [1] as well as of some membrane proteins [2,3]. According to the signal hypothesis [1,4] nascent chains, destined for transmembrane translocation, recognize ER membranes via a sequence of amino acids (signal peptide) usually located at their N-terminus, thus establishing the ribosome–membrane junction. This junction is then most probably maintained not only by the nascent chain but also by a direct link between the ribosome and specific membrane binding sites [5,6]. Recently, two polypeptides, characteristic of rough microsomal membranes and termed 'ribophorins', have been implicated in the attachment of ribosomes to membranes [7].

Inhibitors of ribosome–membrane interaction would clearly be of great interest, both as a possible means to identify the membrane and/or ribosome sites involved in the binding reaction, as well as as tools to study the fate of secretory or membrane proteins synthesized by detached polysomes. The only such inhibitor reported so far is the triphenylmethane dye, aurintricarboxylic acid (fig.1) [6], which has been shown to interfere with ribosome binding by attaching to membrane sites [8]. In addition, when added to a cell-free read-out amino acid incorporation system programmed by rough microsomes, this inhibitor was found to interfere with the cleavage of the signal peptide from a secretory preprotein, suggesting that direct attachment of ribosomes to membranes is necessary for transmembrane translocation and processing of nascent chains [4]. However, the use of ATA in cell free amino acid incorporation systems is of limited value, because the drug has the disadvantage of being a good inhibitor of protein synthesis (of initiation as well as of elongation, at higher concentrations [9]). Moreover, ATA inhibits numerous enzymes [10]. We have therefore searched for other compounds, which might be more selective in their action. Here we report our results with methylenedisalicylic acid, a compound with two aromatic rings, whose condensation with salicylic acid yields ATA (fig.1).

Abbreviations: ATA, aurintricarboxylic acid; ER, endoplasmic reticulum; MDA, dimethyl 5,5-methylenedisalicylic acid; POPOP, 1,4-bis [2-(5-phenyloxasolyl)] benzene; PPO, 2,5-diphenyloxasole; TKM, 25 mM KCl, 50 mM Tris–HCl, (pH 7.5), 5 mM $MgCl_2$

2. Materials and methods

2.1. *Materials*

The following chemicals were purchased from the indicated sources: [^{35}S]methionine (spec. act. 700–

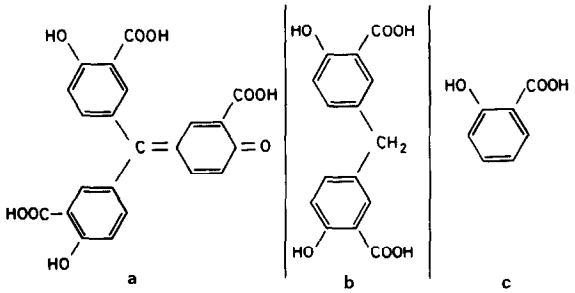

Fig.1. Structural formulae of ATA (a), MDA (b) and salicylic acid (c). Reaction of salicylic acid with MDA in the presence of sodium nitrite and sulfuric acid yields ATA [19].

1100 Ci/mmol) and [5-^{3}H]orotic acid (20–30 Ci/mmol), the Radiochemical Centre (Amersham/Searle); micrococcal nuclease and all reagents for cell free amino acid incorporation, Sigma Chemical Co. (St Louis, MO); ATA, practical grade, Eastman (Rochester, NY); MDA, ICN Pharmaceut. (Plainview, NY); oligo (dT)-cellulose, Collab. Res. (Waltham, MA). All other reagents, of analytical grade, were purchased from Merck (Darmstadt). Centrifugations were carried out in Beckman centrifuges and radioactivity determinations in an Intertechnique SL-30 scintillation counter.

2.2. *Cell fractionation*

Rat liver rough microsomes were prepared as in [11], and the membrane-bound ribosomes were removed by the KCl–puromycin procedure [5,6]. The resulting degranulated rough microsomes were stored in 66% glycerol at –20°C. To label ribosomal RNA, rats were injected with [^{3}H]orotic acid (100 μCi/100 g body wt) and sacrificed 2 days later. The ^{3}H-labeled membrane-bound ribosomes were detached from membranes and collected as in [6]. ^{3}H-Labeled ribosomes and degranulated rough microsomes were used for the cell-free ribosome binding assay (see below).

Free polyribosomes, used for cell free amino acid incorporation, were prepared as in [11,12]. The polysomes were dissociated with SDS (1.5%), the RNA extracted with phenol/chloroform and mRNA purified by oligo(dT) affinity chromatography [14].

2.3. *Binding of ^{3}H-labeled ribosomes to microsomal membranes*

Ribosome-membrane binding was assayed essentially as in [6]. ^{3}H-Labeled ribosomes (5000 dpm ≃ 15 μg RNA) and degranulated rough microsomes (~0.5 mg protein) were incubated for 30 min at 0°C in 0.25 M sucrose–TKM in 0.120 ml final vol. in the absence or presence of inhibitors. The samples were then diluted with 9 vol. 2.2 M sucrose–TKM and overlaid with a discontinuous sucrose gradient consisting of 3.3 ml 1.6 M sucrose–TKM and 1 ml 0.25 M sucrose–TKM. Membrane-attached ribosomes were separated from unattached ribosomes by centrifugation at 45 000 rev./min for 45 min in the SW 50.1 rotor. The membranes at the 1.6–0.25 M sucrose interface were collected and precipitated with trichloroacetic acid. The precipitates were solubilized with Protosol (New England Nuclear) and counted in 10 ml toluene–PPO–POPOP.

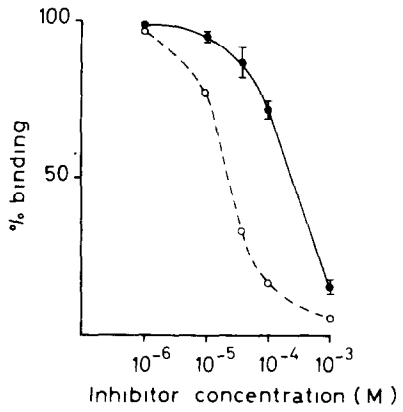

Fig.2. Inhibition of binding of ribosomes to degranulated rough microsomes by ATA and MDA. 100% binding represents binding in controls without added inhibitor (≃3000 dpm). (o–––o) ATA; (•——•) MDA. Bars represent standard errors. For experimental details, see section 2.

2.4. *Cell free amino acid incorporation*

Cell free amino acid incorporation, carried out in a rabbit reticulocyte lysate treated with micrococcal nuclease [15] and desalted by passage through a Sephadex G-50 column, was allowed to proceed at 29°C. At the times indicated in fig.2, 2.5 μl aliquots of the incubation mixtures were pipetted onto Whatman 3 MM filters, and the radioactivity incorporated into protein was determined as in [16].

3. Results and discussion

The inhibitory effects of MDA and ATA on the binding of ribosomes to microsomal membranes are compared in fig.2. The data with ATA are in agreement with those in [8]. As can be seen from the dose–response curves of the two compounds, MDA is ~10-fold less potent than ATA and strongly inhibits the binding reaction (85%) at 1 mM. At this concentration the compound was found to have essentially no effect on protein synthesis, when either rat liver free polyribosomes (fig.3a) or purified mRNA (fig.3b) were used for programming a nuclease-treated rabbit reticulocyte lysate. In the latter case, a slight reduction in the rate of amino acid incorporation was observed, but the final amount of [^{35}S]methionine

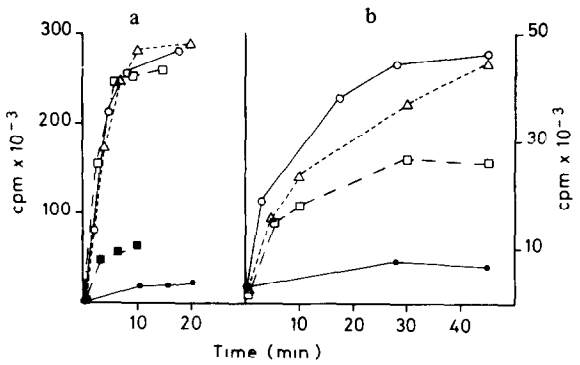

Fig.3. Effect of MDA and ATA on cell-free amino acid incorporation in a nuclease-treated reticulocyte lysate. (•——•) in (a,b) represents time course of incorporation in the lysate without added RNA. All other samples contained: (a) free polyribosomes (1.7 A_{260} units/100 μl incubation mixture); or (b) mRNA purified from free polyribosomes (0.12 A_{260} units/100 μl incubation mixture). (○——○) no inhibitors present; (△– – –△) + MDA 1 mM; (□–·–·–□) + ATA 50 μM; (□–·–·–□) + ATA, 0.5 mM. Each 100 μl reaction mixture contained 25 (a) or 50 (b) μCi [^{35}S]methionine. Radioactivity measurements were carried out on 2.5 μl aliquots.

incorporated was not appreciably different from that of the control sample (fig.3b). The lack of effect of MDA on protein synthesis is in agreement with [17]. In contrast to MDA, concentrations of ATA which inhibit the binding of ribosomes to membranes, also affected protein synthesis. Thus, 50 μM ATA, which inhibits binding by 65%, was inhibitory in the system programmed with mRNA (fig.3b), but had no effect on polysomes (fig.3a). Since ATA at this low concentration is known to be inhibitory on chain initiation, but not on elongation [9], these data suggest that the polysome-programmed system mainly terminates already partially-synthesized peptide chains, but does not initiate the synthesis of new ones. Higher concentrations of ATA (0.5 mM), which inhibit ribosome–membrane interaction by 90%, are known also to block chain elongation [9], and were in fact found to be inhibitory on the polysome-programmed system (fig.3a).

Our results show that MDA, 1 mM, can be used to effectively inhibit the attachment of ribosomes to membranes without interfering with protein synthesis. It is interesting that while all three salicylate rings of the ATA molecule are required for the inhibitory effect on protein synthesis, two rings are sufficient to interfere with ribosome–membrane interaction, although the two-ring compound, MDA, does have a lower potency than ATA. The one-ring compound, salicylic acid, whose condensation with MDA yields ATA, is ineffective as inhibitor both of protein synthesis [16] and of the binding reaction (unpublished).

It must be mentioned that, since the commercial preparations of ATA and MDA are not pure, their inhibitory action on ribosome–membrane interaction might be due not to the compounds themselves, but to contaminants. A crude ATA preparation was fractionated into 8 components and the induction of nuclear swelling and dissociation of histones from DNA characteristic of crude ATA shown to be most probably due to a contaminant, formaurindicarboxylic acid [18]. In view of this possibility, we purified the commercial preparation of MDA, as in [9]. The results obtained with the purified preparation were not different from those shown here. Thus, the inhibitory effect on ribosome–membrane interaction seems to be due to MDA itself, unless the responsible contaminant is not removed by the purification procedure we employed.

In conclusion, MDA promises to be a useful compound in studies aimed at elucidating the role of the ribosome–membrane junction in the processing and transmembrane transfer of secretory and membrane proteins.

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