Translocation of acylated pardaxin into cells

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Abstract Acylated pardaxin is translocated through the cytoplasmic membrane and is accumulated in the nucleoli of NG108-15 and chromaffin cells. The uptake is time- and dose-dependent and temperature-sensitive. However, the binding of acylated ^{125}I-pardaxin cannot be reduced by competition with pardaxin acylated with Rudinger's reagent. In this respect, acylated pardaxin resembles the Tat protein 37–71 fragment. Metabolic inhibitors do not significantly reduce the uptake of acylated ^{125}I-pardaxin. Acylated pardaxin might be useful as a vector to translocate other molecules.

Key words: Pardaxin uptake; Peptide uptake

1. Introduction

The soles of the genus *Pardachirus* Gunther exude secretions into the sea water that are toxic to other fish [1,2] and repel sharks [3–5]. The secretions contain ichthyotoxic peptides named pardaxins [6]. So far, five pardaxins have been identified and sequenced. Three were found in the species *Pardachirus pavoninus* [7] and another two in *Pardachirus marmoratus* [8,9]. All pardaxins are composed of 33 amino acids and form an N-terminal hydrophobic helix (residues 2–10) linked by Ser-Pro to a C-terminal amphiphilic helix (residues 13–27). In aqueous solutions, pardaxin peptides were found in both monomeric and oligomeric forms [10–12]. When pardaxin monomers are inserted into artificial phospholipid membranes or into plasma membranes, they assemble like barrel staves, forming pores with low ionic selectivity [10–19]. Cytolysis follows the pore formation [10]. Acylation of the N-terminal amino group at Gly^1 leaves intact the insertion into membranes but reduces the cytolytic (i.e. hemolytic) activity more than 70-fold [20]. A virtually complete loss of hemolytic activity occurs after acylation of pardaxin with acetic anhydride [21] or Rudinger's reagent [9]. The present experiments originated from our observation that acylated pardaxin reduces the permeability of NG108-15 cells (Fig. 1). So we considered that acylated pardaxin may be bound by and then translocated through cytoplasmic membranes, and that therefore pardaxin might be useful as a vector to translocate other molecules. Here we present data on the binding and translocation of acylated pardaxin.

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Abbreviations: BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5- (and -6-)-carboxyfluorescein acetoxymethylester; Bolton-Hunter reagent, succinimidyl-3-(3-^{125}I-4-hydroxy)phenylpropionate; HPLC, high performance liquid chromatography; HPP, 4-hydroxyphenylpropionyl; ITH, iminothiolane; MCC, 4-(*N*-maleimidomethyl)cyclohexane-1-carboxyl; PDP, 6-[3′-(2-pyridyldithio)propionamido]hexanoyl; PYR, pyrenyldiazomethane; SHPP (Rudinger's reagent), sulfosuccinimidyl-3-(4-hydroxy)phenylpropionate; slcSPDP, sulfosuccinimidyl-6-[3′-(2-pyridyldithio)propionamido]hexanoate; sSMCC, sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate

2. Materials and methods

Bovine chromaffin cells were prepared from suprarenal glands removed from calves within 5 min after slaughter. The source culture of NG108-15 neurohybridoma cells was a gift of Dr. W. Klee, NIMH, Bethesda, MD, USA. T25adh cells were derived from S-49 mouse lymphoma cells [22,23]. The cells were prepared and maintained as described (lymphoma cells [23], chromaffin cells [24], NG108-15 cells [25]). Leu^5-Leu^{14}-Asp^{31}-pardaxin (P5 [9]), GFFALIPKIISSPLFKTLLSAVGSALSSSGDQE, was either purified from the secretion of *Pardachirus marmoratus* or synthesized as described [9]. Succinimidyl-3-(3-^{125}I-4-hydroxy)phenylpropionate (Bolton-Hunter reagent) was from Amersham (Little Chalfont, UK); iminothiolane (ITH), sulfosuccinimidyl-3-(4-hydroxy)phenylpropionate (Rudinger's reagent, SHPP), sulfosuccinimidyl-6-[3′-(2-pyridyldithio)propionamido]hexanoate (slcSPDP) and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC) were from Pierce (Rockford, IL); 2′,7′-bis-(2-carboxyethyl)-5- (and -6-)carboxyfluorescein acetoxymethylester (BCECF-AM), pyrenyldiazomethane (PYR), and monosuccinimidyl Cy3 were from Molecular Probes (Eugene, OR, USA). All other chemicals and the culture media were of the highest quality available.

2.1. Synthesis of Cy3-pardaxin

The lyophilized content of one vial of monosuccinimidyl-Cy3 (about 250 μg or 326 nmol) was dissolved in 184 μl of a 0.1 M sodium bicarbonate solution containing 184 μg (54 nmol) pardaxin. The solution was agitated overnight. The fluorescent reaction product was purified by HPLC on a Vydac C_{18} reverse-phase column (250×4.6 mm, 5 μm, 300 Å) [9].

2.2. Synthesis of HPP-pardaxin

Pardaxin (53.2 nmol) was reacted overnight with SHPP (1.07 μmol) in 103 μl 0.1 M borate buffer, pH 8.5. The reaction product was purified by HPLC.

2.3. Synthesis of ^{125}I-HPP-pardaxin

One nanomol pardaxin, dissolved in 60 μl of 0.1 M borate, pH 8.5, was added to 0.5 nmol (1 mCi) of Bolton-Hunter reagent. The reaction vessel was rotated. After 1 h, 20 nmol of SHPP in 10 μl borate buffer was added to acylate the remaining primary amino groups. Three days were allowed for the hydrolysis of the remaining free succinimidyl groups of SHPP not coupled to pardaxin.

2.4. Synthesis of MCC-pardaxin and PDP-pardaxin

The reaction mix contained 4 mM pardaxin and 40 mM (in two syntheses 80 mM) sSMCC or slcSPDP, respectively, in DMSO. The

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reaction time was at least 3 h. The ensuing MCC-pardaxin or PDP-pardaxin derivatives were purified by centrifugation through Sephadex G-25 F swollen either in PBS or 0.1 M borate buffer at pH 8.5. In the PBS filtrates, they were deactivated with mercaptoethanol or cysteine. In the borate filtrates, they were mixed with iminothiolane-thiolated proteins (transferrin or human IgG). At least one day was allowed for the protein-disulfide exchange between PDP-pardaxin and thiolated protein. The pardaxin-protein (i.e. IgG, transferrin) conjugates were purified by centrifugation through Sephadex G-25 F swollen in PBS.

2.5. Synthesis of PYR-pardaxin

Equal volumes of pardaxin 2.5 mM in DMSO and PYR 2.5 mM in DMSO were mixed and allowed to react for 2 h. The reaction mix was centrifuged through Sephadex G-25 F swollen in DMSO. The fluorescent PYR labeled pardaxin at its carboxyl groups. In the syntheses of the pardaxin-protein conjugates, PYR-pardaxin was added to pardaxin to permit the determination of the pardaxin/protein ratio. The concentration of PYR-pardaxin was 1/100 of the pardaxin concentration.

2.6. Light microscopic visualization of cells incubated with Cy3-pardaxin

The cells were plated at a density of 10^5 cells/200 mm^2 on cover glasses coated with collagen. NG108-15 cells were incubated for 4 h and chromaffin cells for 16 h with Cy3-pardaxin. Two concentrations (25 or 100 nM) were used. All cells were then washed twice with PBS, fixed with 4% buffered formaldehyde for half an hour, washed twice with PBS, covered with glycerol and stored at 4°C. Normal fluorescence and Nomarski photography was done with a Zeiss Axiovert microscope.

2.7. Confocal microscopy

A Bio-Rad MRC-1024 confocal scanhead coupled to a Zeiss Axiovert 135M inverted microscope was used to acquire images of the stained cells, with a 63× oil objective (numerical aperture 1.4). Excitation light was provided by a 100 mw air-cooled argon ion laser run in the multi-line mode. The cells were labeled with Cy3-pardaxin. The data presented in this report were obtained using 514 nm excitation. The emission filter in the Cy3 detection channel was either a 585LP low pass filter (585 nm, 50% point) or an HQ570/30 (570 ± 15 nm) interference filter. The iris aperture was 2.5 mm. In addition, the emission was detected simultaneously in a second channel, using either a 540/30 bandpass filter (540 ± 15 nm) or an HQ535/20 bandpass filter (535 ± 10 nm). This channel was used to provide an internal control against autofluorescence, as explained below. Images of 512×512 pixels were acquired, and a 3×3 median filter was used to remove point noise from the digital images. A linear contrast stretch was applied to enhance contrast. Unstained controls were found to produce a green/yellow autofluorescence when excited at 488 nm. This autofluorescence decreased by at least a factor of 4 when excited with 514 nm under identical detection conditions. In order to determine that the emission which was observed was due primarily to Cy3, the images were obtained both at 488 nm and 514 nm in both emission channels. The short wavelength channel was dominated by autofluorescence, while the long wavelength channel detected Cy3 much more efficiently, and perhaps also autofluorescence. In order to rule out autofluorescence, the images obtained at 514 nm were compared to those obtained using 488 nm. At 514 nm, the response of the short wavelength channels decreased, while the long wavelength channel either remained unaffected or showed some increase in signal. Although it is possible that Cy3 emission bled through the 540/30 filter, this channel still provided an upper limit on autofluorescence. Thus, while it is likely that some autofluorescence is detected (at least through the HQ570/30 filter), it represents only a small part of the detected signal.

2.8. Binding and translocation studies

NG108-15 cells were plated on 24-well plates (10^5 cells/well) and maintained for two days at a low (1%) FCS concentration. They were incubated with various solutions at 37°C or 0°C for 2 h. A combination of deoxy-D-glucose 25 mM, NaCN 10 mM and NaN_3 100 mM in isotonic saline was used for metabolic inhibition. After incubation, the cells were washed twice with cold PBS, harvested and centrifuged. The pellet was again washed with cold PBS and was counted in a gamma counter.

2.9. Permeability of the cytoplasmic membrane

NG108-15 cells were plated on 24-well plates (10^5 cells/well) and were maintained at a 1% FCS concentration for two days. The content of a BCECF-AM vessel (50 μg) was dissolved in 30 μl DMSO and further diluted into 6.1 ml serum-free equilibrated DBME. The cells were washed twice with this medium, covered with 250 μl BCECF-AM dilution/well and returned to the incubator. After 30 min, they were washed twice with 37°C HEPES medium (25 mM HEPES, 110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 25 mM glucose, adjusted to pH 7.5 with NaOH), covered with pardaxin or pardaxin derivatives dissolved in HEPES medium (500 μl/well), and returned to the incubator for 15 min. The supernatants were then removed and pressed through glass filters. The filtrates were diluted 1+4 with HEPES medium, the fluorescence of the dilutions was excited at the 505-nm optimum and at the 439-nm isosbestic point, and was recorded at 535 nm. The isosbestic correction had virtually no influence on the results.

2.10. Cytolysis

T25adh cells were plated on 24-well plates. Two hours later, the cells were incubated with pardaxin or one of its derivatives. After 45 and 120 min of incubation, a person not knowing the protocol ranked the degree of damage to the cells into five categories.

2.11. Peptide and protein determination

The peptide content of the pardaxin derivative solutions and the protein content of cell solubilisates were determined with the Sigma BCA assay, using as a standard either synthetic pardaxin (for the pardaxin derivatives) or BSA.

3. Results

3.1. Action on the permeability of cytoplasmic membranes

At low concentrations, pardaxin and acyl-pardaxin decreased the spontaneous leakage of fluorescent BCECF from

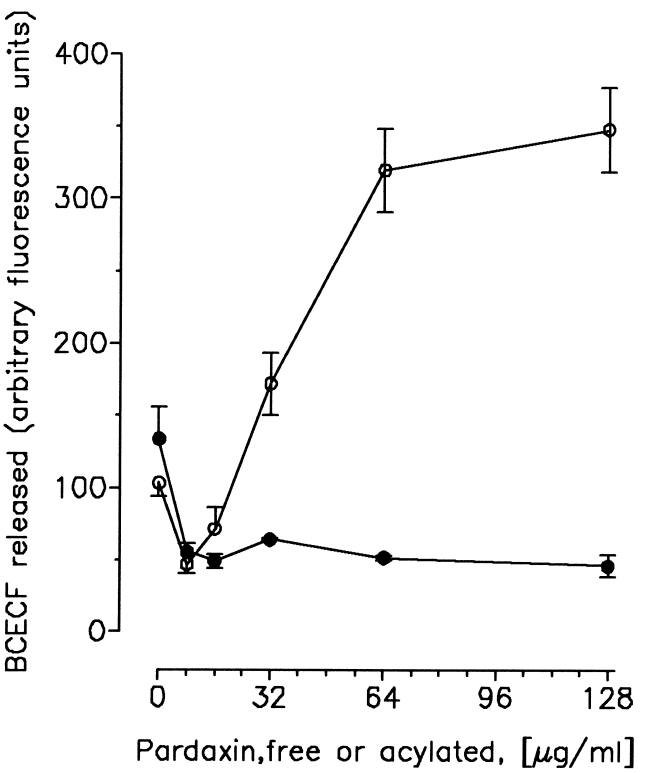

Fig. 1. Action of pardaxin (open circles) and acylated pardaxin (filled circles) on BCECF release from NG108-15 cells.

NG108-15 cells (Fig. 1). At elevated concentrations, only pardaxin increased the BCECF leakage.

3.2. Intracellular detection of Cy3-pardaxin

With confocal microscopy, Cy3-pardaxin was detected inside NG108-15 cells (Fig. 2) and inside chromaffin cells (not shown). After 4 h of incubation, the intensity of fluorescence was smaller in chromaffin cells than in NG108-15 cells. The glia cells had a stronger fluorescence than the chromaffin cells. Structural elements (nucleoli) inside the nuclei were heavily labeled. This strong fluorescence was consistently found in several cells of the same cover slip as well as in cells from cover slips of another experiment. On scanning, fluorescent spots were seen in the cytoplasm, some of which had a vesicular shape. Fluorescence of the plasma membrane was obvious particularly in the top sections, where the membrane looked like a fluorescent cap. Fluorescent 'parcels' could be seen in the dendrites of NG108-15 cells. NG108-15 cells from the same cover slip showed different intensities of fluorescence. No cell damage exceeding the controls was detected in either the NG108-15 or the chromaffin cells.

3.3. Binding and translocation

At 37°C, binding plus translocation of ^{125}I-HPP-pardaxin was dose-dependent (Fig. 3) and reached equilibrium after more than 2 h. However, in a competitive experiment, this accumulation was not reduced with the 1000-fold concentration of 'cold' HPP-pardaxin (data not shown). Binding plus translocation of acylated ^{125}I-pardaxin was time-dependent and was much lower at 0°C than at 37°C (Fig. 4), and metabolic inhibitors did not significantly reduce its uptake (data not shown).

3.4. Cytolysis

Pardaxin at a concentration of 20 mM produced complete cytolysis in T25adh-cells. Pardaxin acylated with either slcSPDP or sSMCC and then deactivated with mercaptoethanol had lost its cytotoxicity (data not shown).

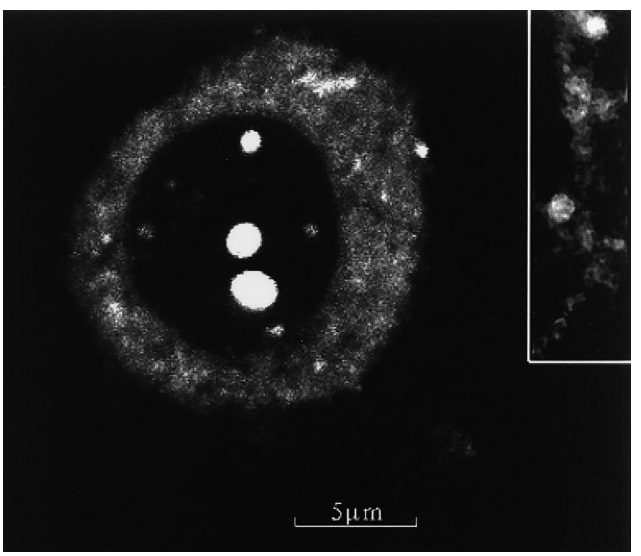

Fig. 2. Detection of Cy3-acylated pardaxin in NG108-15 cells. Beginning at the bottom of the cell, confocal sections were acquired using half micron steps. Sections 8–12, acquired over a depth of about 2.5 µm, were projected onto the same plane to show several nucleoli which are not all in the same plane. The inset shows a single section through a dendrite of another cell.

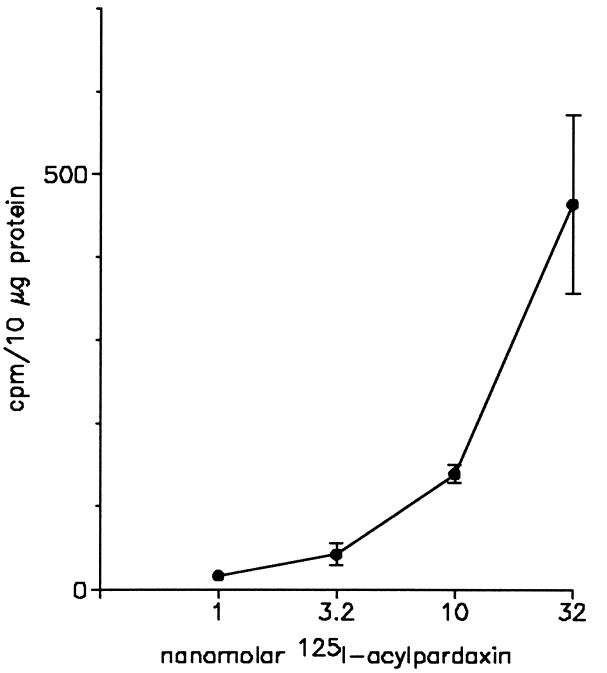

Fig. 3. Dose-dependent binding plus uptake of acylated ^{125}I-pardaxin by NG108-15 cells at 37°C.

4. Discussion

Acylated pardaxin is not only inserted into cytoplasmic membranes [10,17], but is translocated and accumulated in the nucleus. The binding of acylated pardaxin is not a classical high affinity receptor binding because no binding constants or number of binding sites could be determined with a Scatchard experiment. However, this distinctive kind of non-competitive peptide binding has already been described for the Tat protein 37–72 fragment [26] and for the *Drosophila antennapedia* homeodomain (Antp-HD, [27]). The binding of the ^{125}I-Tat fragment is even increased by cold Tat fragment. In contrast, acylated pardaxin does not increase the binding of acylated ^{125}I-pardaxin. The translocation of acylated pardaxin is temperature-sensitive. The fluorescent vesicular structures appearing in the cytoplasm of NG108 as well as of chromaffin cells point to an uptake via endocytosis or pinocytosis, but the binding experiments as well as the experiments with metabolic inhibitors lend no support to this hypothesis. Therefore, the uptake mechanism and the vesicle structure need further characterization. Because both the Tat peptide fragment and the Antp-HD are translocated, they are used for the introduction of other peptides, proteins, and oligonucleotides [28,29]. For the same reason, pardaxin derivatives might be useful as vectors to translocate other molecules.

The cytolytic activity of pardaxin was lost after acylation of the amino group of Gly^1 [20] or of the amino groups in Gly^1, Lys^8 and Lys^{16} [21] with the small succinic anhydride molecule. The present experiments provide additional evidence that this is due to the acylation of the amino groups rather than to the introduction of free carboxyl groups with succinic anhydride: upon acylation with any of the reagents Cy3, Cy3.5, sSMCC, slcSMCC (this paper) or SHPP [9], or on tri-acyla-

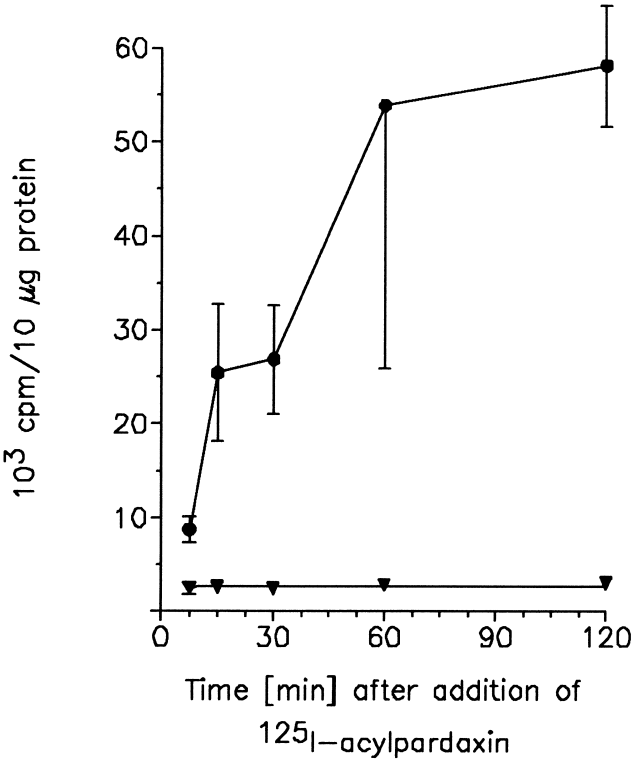

Fig. 4. Time-dependent binding plus uptake of 10 nM acylated ^{125}I-pardaxin by NG108-15 cells at 37°C (filled circles) and 0°C (filled squares).

tion (Table V in [21]), pardaxin always lost its cytotoxic activity.

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