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Perturbations of model membranes induced by pathogenic dynorphin A mutants causing neurodegeneration in human brain

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ABSTRACT

Several effects of the endogenous opioid peptide dynorphin A (Dyn A) are not mediated through the opioid receptors. These effects are generally excitatory, and result in cell loss and induction of chronic pain and paralysis. The mechanism(s) is not well defined but may involve formation of pores in cellular membranes. In the 17-amino acid peptide Dyn A we have recently identified L5S, R6W, and R9C mutations that cause the dominantly inherited neurodegenerative disorder Spinocerebellar ataxia type 23. To gain further insight into non-opioid neurodegenerative mechanism(s), we studied the perturbation effects on lipid bilayers of wild type Dyn A and its mutants in large unilamellar phospholipid vesicles encapsulating the fluorescent dye calcein. The peptides were found to induce calcein leakage from uncharged and negatively charged vesicles to different degrees, thus reflecting different membrane perturbation effects. The mutant Dyn A R6W was the most potent in producing leakage with negatively charged vesicles whereas Dyn A L5S was virtually inactive. The overall correlation between membrane perturbation and neurotoxic response [3] suggests that pathogenic Dyn A actions may be mediated through transient pore formation in lipid domains of the plasma membrane.

1. Introduction

Prodynorphin (PDYN), a precursor protein to endogenous opioid peptides gives rise to α-neoendorphin, dynorphin A (Dyn A) and dynorphin B (Dyn B), endogenous ligands for the κ-opioid receptor. In addition to opioid actions, Dyn A but not the other PDYN derived peptides may induce several pathological effects that are not mediated through the opioid receptors, such as neurological dysfunctions and cell death [1,2]. For example, intracerebroventricular injection of this peptide induces abnormal motor effects consisting of e.g. wild-running, barrel-rolling, and ataxia in mice, while intratechal infusion produces allodynia, paralysis and neuronal loss in rats [1,3]. Upregulation of Dyn A in the spinal cord underlies maintenance of neuropathic pain [1,4], while pathological pain may be induced at femto-molar doses of intratechal Dyn A [4].

Spinocerebellar ataxia (SCA) is a dominantly inherited neurodegenerative disease characterized by progressive cerebellar ataxia, dysarthria and oculomotor abnormalities [5]. The SCAs form a genetically heterogeneous group of ataxias that result from atrophy of selective neurons in the cerebellum and spinal cord [5,6].

Recently, four missense mutations were identified in the *PDYN* gene to cause SCA23 [3]. Interestingly, three out of four mutations were located in the part of *PDYN* that encodes for Dyn A, a peptide with opioid and non-opioid pathogenic activities (Table 1). Model experiments demonstrated that the Dyn A L5S and R6W mutations in the 17-amino acid Dyn A result in 20–40-fold elevation of the concentration of the neuropeptide Dyn A compared to concentrations of Dyn B and Leu-enkephalin-Arg [3]. This may be due to slow or aberrant conversion of Dyn A mutants to enkephalins as two of its crucial arginines required for proper processing are mutated. Analysis of the effect of mutant Dyn A peptides on striatal neurons demonstrated an elevated 2–3-fold toxicity of Dyn A R6W and R9C compared to the wild type (WT) [3]. This observation suggests a dominant negative effect of these mutations rather than a loss of function.

The mechanisms underlying the non-opioid pathogenic Dyn A actions are not well defined but have been proposed to involve interactions of these peptides with the plasma membrane, such as peptide translocation into the cell [7] and/or formation of

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Table 1
Summary of the physico-chemical properties of Dyn A WT and its three mutants together with results of their interactions with uncharged and negatively charged LUVs as well as cell toxicity data.

Peptide	Dyn A (WT)	Dyn A (R6W)	Dyn A (R9C)	DynA (L5S)
Sequence	YGGFLRRIRPKLKWDNQ	YGGFLWRIRPKLKWDNQ	YGGFLRRICPKLKWDNQ	YGGFSRRIRPKLKWDNQ
Average hyrophobicity[a]	1.40	1.26	1.24	1.48
No. of Arginines in sequence	3	2	2	3
POPC % membrane leakage[b]	37	27	22	0
70/30 POPC/POPG % membrane leakage[b]	19	59	11	0
Peptide induced cell death[c]	1.5	3	2.5	<0.1

[a] Average hydrophobicity was calculated utilizing the von Heijne scale for biological hydrophobicity [19].
[b] Dyn A WT and its mutants were titrated to calcein-entrapping POPC and 30% negatively charged POPC/POPG LUVs, and calcein release recorded after 10 min incubation. The uncertainty at this time point is about 5%.
[c] Peptide induced cell death, relative units. One hundred nanomolar peptide was applied to striatial neurons and cell viability was recorded after 60 h. Data from [3], Fig. 2F.

membrane pores allowing influx of calcium ions into the cell [8]. Such calcium influx may produce excitotoxic response, neuronal dysfunction and atrophy. In this study, to gain better insight into the non-opioid mechanism, we have compared the potency to induce membrane perturbations between Dyn A WT and the pathogenic mutants. As a model membrane system we used large unilamellar vesicles (LUVs) with the encapsulated fluorescent dye calcein, which allows monitoring of membrane perturbations using fluorescence spectroscopy [9,10].

2. Materials and methods

2.1. Materials

Zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-rac-(1-glycerol)] with a negative headgroup at neutral pH (POPG) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA) and were used without further purification. PD-10 desalting columns were obtained from GE Healthcare (Buckinghamshire, UK). Calcein, a fluorescein derivative ($C_{30}H_{26}N_2O_{13}$, 622.5 Da, Molecular Probes) was obtained from Molecular Probes, the Netherlands. Dyn A(1–17) wild type (YGGFLRRIRPKLKWDNQ) and the three mutants Dyn A R6W, R9C, and L5S were synthesized at the Leiden University Medical Center, Leiden, The Netherlands, purified by reversed-phase and Superdex column chromatography, and analyzed by analytical reversed-phase chromatography and matrix-assisted laser desorption ionization-time of flight mass spectrometry. The purity of all peptides was ~98%.

2.2. Determination of peptide concentration

The concentration of the peptides was determined in aqueous solution with a CARY 4 UV/Vis spectrophotometer by measuring the optical absorption at 280 nm. Molar absorption coefficients of 5690 M^{-1} cm^{-1} and 1280 M^{-1} cm^{-1} at 280 nm were used in the concentration calculations for one Trp and one Tyr, respectively.

2.3. Preparation of large unilamellar vesicles

Large unilamellar vesicles (LUVs) were prepared by dissolving the phospholipids (zwitterionic POPC and 30% negatively charged POPG) in chloroform to obtain a homogeneous solution, whereafter the solvent was removed by evaporation under high vacuum for 3 h. The resulting dried lipid film was resuspended by addition of 50 mM potassium phosphate buffer at pH 7.2. This solution was vortexed for 10 min and then subjected to five freeze–thaw cycles with liquid nitrogen to reduce the lamellarity. Next, an Avanti manual extruder was used to push the lipid solution 20 times through two polycarbonate filters (100 nm pore size). This method gave unilamellar vesicles with 100 nm diameter [11], and the size and stability of the LUVs were examined with a dynamic light scattering (DLS) instrument.

2.4. Calcein leakage experiment

LUVs with entrapped calcein were prepared as described above, using a buffer solution containing 55 mM calcein at pH 7.2. Calcein not entrapped inside the LUVs was removed by filtering the LUV solution through Sephadex-G25 columns two times [9]. The fluorescence intensity of calcein was recorded on a Horiba Jobin Yvon Fluorolog-3 spectro-fluorometer with wavelengths set at 490_{ex} and 516_{em} nm. The fluorescence intensity for a solution of 55 mM calcein should be low due to self quenching, and increase upon dilution. After measuring the background intensity, Dyn A peptides in concentrations of 2, 5, 10 and 15 μM were added to the vesicle solution, which either consisted of 100 μM POPC or 100 μM POPC/POPG (70%/30%) at 25 °C. The release of calcein from the vesicles was then monitored over 10 min as an increase in the fluorescence intensity. In the final step one hundred percent leakage (i.e. destruction of the vesicles) was induced by addition of 10% (w/v) Triton X-100 (Sigma–Aldrich, Stockholm, Sweden). Using this limit as an upper reference level and the background fluorescence as a lower reference level, the degree of leakage induced by different Dyn A peptides was calculated using the following equation:

$$\% \text{ leakage} = [(F - F_0)/(F_r - F_0)] \times 100$$

where F_0 and F_r are the respective fluorescence intensities initially observed without peptide (background) and after treatment with Triton X-100. F is the fluorescence intensity in the presence of Dyn A peptides. The experiments were performed three times, and in all experiments the calcein leakage was almost fully saturated after 10 min incubation. The standard deviation at the endpoint (10 min) was approximately ±5 in units of percent calcein release (Fig. 1).

3. Results

The amino acid sequences of Dyn A WT and its mutants Dyn A R6W, L5S and R9C are shown in Table 1 together with data showing their average hydrophobicity and number of arginines. The membrane perturbation effects of the different peptides were investigated by studying calcein release from 30% negatively charged LUVs and from zwitterionic POPC LUVs. In the absence of peptides, no leakage of calcein from the LUVs was observed. The fluorescence intensity in this case was low due to the self-quenching of calcein inside the LUVs. Upon addition of the peptides to the LUVs, the entrapped calcein is typically released into the buffer outside the LUVs as a result of induced leakage, leading to an increase in fluorescence intensity.

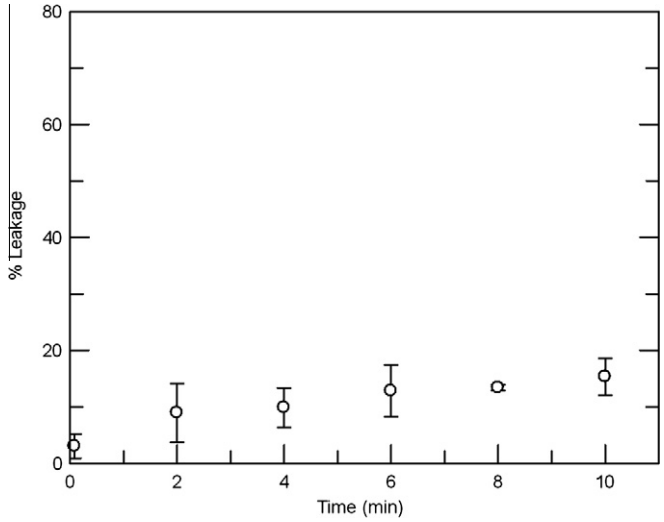

Fig. 1. Time dependence of calcein leakage after addition of 10 μM Dyn A WT to a vesicle solution containing 55 mM entrapped calcein inside 30% negatively charged LUVs composed of POPC and POPG (7:3) with total lipid concentration of 100 μM at 25 °C. The standard deviation based on three repeats is about 5%.

In the titrations, fluorescence intensities were recorded during 10 min incubation. Fig. 1 shows the time dependence of calcein release after addition of 10 μM DynA WT to 30% negatively charged LUVs. Figs. 2 and 3 show the amount of leakage induced by the peptides in 30% negatively charged and in zwitterionic LUVs respectively, after 10 min incubation at 25 °C, as a function of peptide concentration. In the presence of negatively charged LUVs, Dyn A WT, Dyn A R9C, and in particular Dyn A R6W induced a substantial degree of calcein leakage. In the presence of zwitterionic POPC LUVs, Dyn A WT induced a higher degree of leakage than all three mutants. In contrast, Dyn A L5S induced no leakage at all, neither in the negatively charged nor in the uncharged vesicles. Table 1 summarizes the leakage results observed for the different peptides in the two types of LUVs and also includes cell toxicity data reported previously [3].

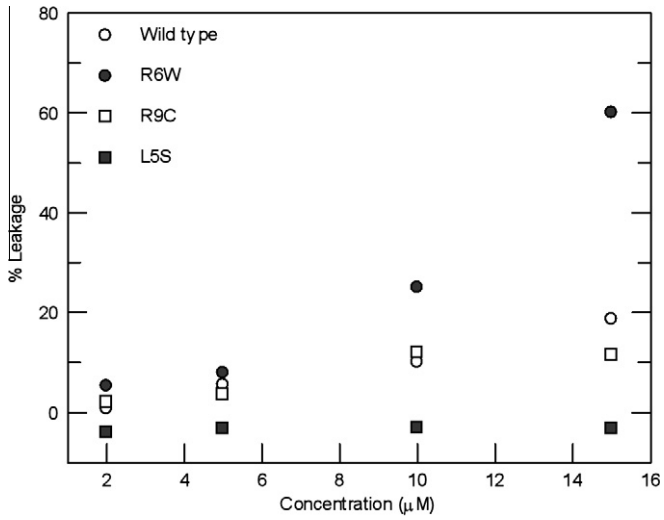

Fig. 2. Calcein leakage experiment with Dyn A WT (open circles) and the three mutants Dyn A R6W (solid circles), Dyn A R9C (open squares), and Dyn A L5S (solid squares), titrated to a vesicle solution containing 55 mM entrapped calcein inside 100 μM negatively charged POPC:POPG 7:3 LUVs at 25 °C. The calcein leakage percent was measured 10 min after adding different amount of the peptides, and plotted as a function of peptide concentrations. The negative intensity values for Dyn A L5S correspond to the dilution caused by addition of the peptide.

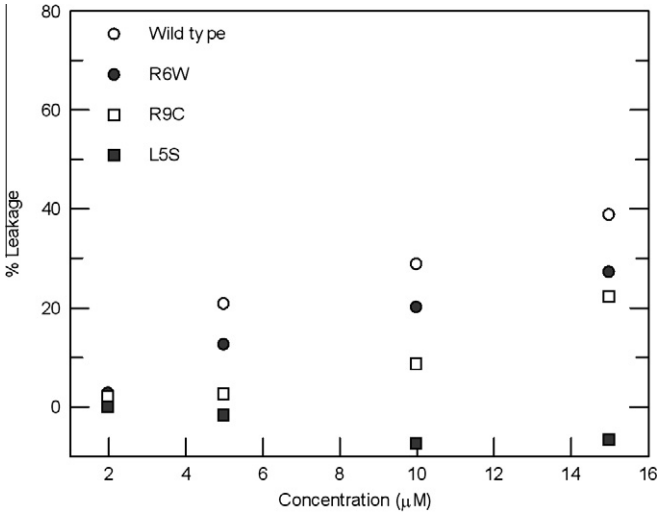

Fig. 3. Calcein leakage experiment with Dyn A WT (open circles) and the three mutants Dyn A R6W (solid circles), Dyn A R9C (open squares), and Dyn A L5S (solid squares), titrated to a vesicle solution containing 55 mM entrapped calcein inside 100 μM zwitterionic POPC LUVs at 25 °C. The calcein leakage percent was measured 10 min after adding different amount of the peptides, and plotted as a function of peptide concentrations. The negative intensity values for Dyn A L5S corresponds to the dilution caused by addition of the peptide.

4. Discussion

In this study, we performed calcein leakage experiments with phospholipid LUVs as membrane models. The aim was to investigate the membrane perturbation effects of Dyn A WT and mutants, to determine the mechanism for their non-opioid activities. LUV calcein leakage studies are indicators of the degree of perturbation to the membrane caused by different peptides, and the results are usually related to transient pore formation [9,12–14]. We used both neutral and partially negatively charged LUVs, as native eukaryotic cell membranes are overall neutral even though they contain about 10% negatively charged lipids, mostly located on the inner leaflet of the membrane bilayer [15]. In addition, negatively charged proteoglycans are present on the cell surface where they contribute to the electrostatic interactions with other molecules [16,17]. We therefore consider both types of LUVs as relevant membrane model systems for eukaryotic cell membranes.

The results of this study (see Table 1) show that Dyn A WT and particularly its mutant R6W induce potent calcein leakage in both the charged and the uncharged model membrane systems. The highest degree of leakage in the charged LUVs is caused by Dyn A R6W, whereas in the uncharged LUVs Dyn A WT is most potent. Dyn A R9C has a lower overall leakage inducing potency than Dyn A R6W and WT. Dyn A L5S does not induce any leakage in any of the vesicle types. Peptides that penetrate or perturb biological membranes are often discussed in terms of hydrophobicity, total charge and number of arginines, but there is no consensus about direct correlations between these parameters and the biological activities [18]. Comparing Dyn A and its mutants (Table 1) we observe that they vary in hydrophobicity (Dyn A WT and R9C are most hydrophobic), as well as total charge and number of arginines. Table 1 shows that the peptides also vary in terms of neurotoxicity [3]. The neurotoxicity index is highest for Dyn A R6W, followed by R9C and WT. Dyn A L5S is not neurotoxic.

The results indicate that there is an overall correlation between membrane perturbation and neurotoxicity, since the neurotoxic Dyn A WT, R6W and R9C peptides induce significant membrane leakage to varying extents in the vesicles, whereas L5S is inactive in both respects. The detailed properties of the peptides causing

leakage in the uncharged membrane model systems show less precise correlation with the biological neurotoxicity results. The membrane leakage in the negatively charged LUVs seems to correlate best with the neurotoxicity results. In conclusion we propose that transient pore formation in phospholipid membranes caused by Dyn A and its mutants is the major cause of their neurotoxic effects.

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