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Inhalable liposomal formulation for vasoactive intestinal peptide

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Abstract

Inhalation of vasoactive intestinal peptide (VIP) was suggested as promising treatment option of various lung diseases like asthma and pulmonary hypertension. However, the medical use of peptides is limited by their short half-life due to rapid enzymatic degradation in the airways. For that reason, we recently developed unilamellar nano-sized VIP-loaded liposomes (VLL). Now we investigated their applicability for inhalation purposes.

After nebulisation by a mouthpiece ventilation inhaler we found the particle size almost unaffected, being in a size range appropriate for bronchiolar deposition; we observed no peptide release due to nebulisation. The VIP release kinetics from VLL were tested by an *ex vivo* vasorelaxation model. Exposure to target organs revealed an immediate response, which was significantly retarded for VLL as compared to free VIP ($p = 0.001$). Using vasorelaxation as endpoint, we observed a sustained release and an extended pharmacological effect compared to equimolar free VIP.

The liposomes have the potential to improve VIP inhalation therapy by providing a "dispersible peptide depot" in the bronchi. Thereby, the release of VIP from liposomes may be triggered by exposure to cells, i.e. directly by ligand–receptor interactions.

Keywords: Nanomedicine; Liposome; Drug inhalation; VIP inhalation

1. Introduction

Many peptides are highly specific signalling molecules, which are effective at low molar concentrations, and are therefore suitable for the treatment of various diseases including lung disorders. However, their pharmaceutical applicability is strongly limited due to the fact that they become rapidly degraded by peptidases. To improve the established therapeutic potential of inhaled vasoactive intestinal peptide (VIP) we have recently designed a new liposomal delivery system (Stark et al., 2007). Now, we investigated its suitability for the use with a commercial mouthpiece ventilation inhaler, and observed the peptide-release kinetics in a physiological *ex vivo* model.

1.1. Vasoactive intestinal peptide

Vasoactive intestinal peptide is a neuro-peptide, discovered in the small intestine (Said and Mutt, 1970). The receptors are expressed by endothelium and smooth muscles of the blood vessel wall (Hamasaki et al., 1983; Saga and Said, 1984), bronchial wall (Groneberg et al., 2001a), and immune cells (Delgado et al., 1999).

The administration of exogenous VIP was described for the treatment of several lung diseases. About 80 peer reviewed publications deal with the therapeutic potential of VIP in asthma, and about 26 of them describe human studies (Said, 1991; Groneberg et al., 2001b). Nevertheless, VIP agonists did not reach clinical use yet (Groneberg et al., 2006), most probably because therapeutically administered VIP is prone to rapid proteolytic digestion and inactivation by endopeptidases, reducing the bioavailability of the peptide (Tam and Caughey, 1990; Hachisu et al., 1991; Lilly et al., 1993).

As VIP inhalation was shown to be effective in idiopathic pulmonary artery hypertension (Petkov et al., 2003; Rubinstein, 2005), pulmonary administration can be considered as an attractive alternative to decrease intravenous VIP degradation.

1.2. VIP receptor recycling

Upon ligand binding, VIP receptors are internalised to the cell cytoplasm. A rapid receptor turnover to the cell membrane,

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which occurs within minutes, was described for rat and human cells (Boissard et al., 1986; Izzo et al., 1991). Following receptor saturation, a rapid receptor turnover allows additional ligands to "wait" for a recycled receptor. Thus, a VIP depot formulation which provides a retarded release of its cargo would protect the peptide while "waiting" for a recycled receptor and thereby, the prolonged local availability of VIP would enhance the therapeutic effect.

1.3. VIP in inhalable liposomes

Liposomes are excellent carriers for therapeutic drugs (Lasic, 1998; Lian and Ho, 2001; Allen and Cullis, 2004; Torchilin, 2005) whereas a protective surface layer formed by a hydrophilic polymer can reduce recognition and clearance by macrophages (Allen and Hansen, 1991; Allen, 1998). Hence, intravenously administered sterically stabilised liposomes can function as efficient substance depots for VIP (Sethi et al., 2005; Önyüksel et al., 2006). Inhalable VIP-loaded liposomes (VLL) can circumvent intravenous administration.

Criteria for successful aerosol delivery are on the one hand the optimal droplet size to reach the lower bronchioles (Labiris and Dolovich, 2003), and on the other hand the stability and size of the nano-carrier system itself.

The association of VIP with phospholipids results in a conformational change of the peptide from random coil to α-helical (Gololobov et al., 1998). This structural modification protects VIP from degradation and inactivation, and in turn is the preferred conformation for ligand–receptor interaction (Sethi et al., 2005).

In a previous study, we have established and characterised a ternary lipid mixture that assembles to form unilamellar liposomes (Stark et al., 2007). Now, we were interested in the potential of this liposomal formulation to be used as inhalable peptide carrier.

1.4. Liposomes for lung delivery

Pulmonary administration of biomolecules appears very promising, offering the advantage of increased drug concentrations at the site of action (Niven, 1995; Agu et al., 2001). Nevertheless, aerosol delivery of peptides faces certain stability problems, which probably could be overcome by the design of proper carrier systems. The use of liposomes for pulmonary drug administration offers the advantage to minimise adverse side effects, to prevent local irritations in the lung, and to show increased potency by exhibiting prolonged release rates (Waldrep, 1998; Cryan, 2005). The suitability of liposomes for inhalation, however, strongly depends on the drug to be delivered as well as on the lipid composition (Niven and Schreier, 1990; Desai et al., 2002), size (Niven et al., 1991) or charge of the liposomal formulation and, finally, on the method of application (Waldrep et al., 1994; Waldrep et al., 1997). Moreover, successful drug delivery might be hampered by disintegration and instability of the liposomes caused by the high shear forces exerted by nebulisers leading to unwarranted drug release (Niven and Schreier, 1990; Niven et al., 1992). In general, drug retention is higher for smaller liposomes, which additionally are less rapidly opsonized than their larger counterparts (Niven et al., 1991). Thus, liposomes below 200 nm in diameter are suggested to be optimal for pulmonary applications (Labiris and Dolovich, 2003).

Different nebulisers have been tested to address their suitability for pulmonary application of liposomes (Waldrep et al., 1994), whereas jet nebulisers have been shown to be most effective for aerosol droplet deposition in lung tissues (Waldrep, 1998; Elhissi et al., 2007). Especially the size of the droplets in which the liposomes are dispersed is an important factor of lung deposition. Aerosol droplets should be less than 5 μm in diameter to be deposited into the lower respiratory tract and even smaller particles of around 3 μm are required for reaching the alveolar system (Labiris and Dolovich, 2003).

The liposomal formulation presented in this paper is specially customized for VIP having a high density of negative surface charges for electrostatic attraction of VIP, thereby inducing a conformational change in the secondary structure of the peptide. Additionally, a PEG-shell is provided to shield surface-attached VIP molecules from enzymatic degradation in the airways and to inhibit particle aggregation. Moreover, the PEG-liposomes have proven to be highly stable during nebulisation with commercially available nebulisers (Anabousi et al., 2006).

2. Material and methods

2.1. Reagents

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). VIP (amino acid sequence HSDAVFTDNYTRLRKQMAVKKYLNSILN-NH_2) was synthesized by piCHEM (Graz, Austria, Europe) using FMOC solid-phase peptide synthesis methodology (FMOC-SPPS). The peptides were purified by RP-HPLC.

All other chemicals were purchased from Sigma–Aldrich (Vienna, Austria, Europe).

Krebs–Henseleit buffer (KH) was used for the physiological experiments. Composition in mM: 118.40, NaCl; 5.01, KCl; 1.20, KH_2PO_4; 2.5, $CaCl_2$; 1.2, $MgCl_2$; 10.1, glucose; 25, $NaHCO_3$.

2.2. Liposome preparation

Empty and passively VIP-loaded liposomes, composed of a ternary mixture of polyethylene glycol-conjugated distearyl phosphatidyl ethanolamine (DSPE-PEG2000), lysostearyl-phosphatidylglycerol (lyso-PG) and palmitoyl-oleoyl-phosphatidylcholine (POPC) with a molar ratio of 5:38:57 mol% were synthesized by thin film rehydration method as described previously (Stark et al., 2007). The thin lipid film was resuspended in 130 mM phosphate buffered saline, pH 7.4 (PBS) or, for peptide loading, in an aqueous solution of 0.5 mg/ml VIP in PBS (molar phospholipid to VIP ratio of 250:1) and hydrated at 40 °C for 1 h by repeated vortexing. The liposomal formulation used in this study is specially customized for VIP having a high density of negative surface charges for

electrostatic attraction of VIP, thereby inducing a conformational change in the secondary structure of VIP. Additionally, a PEG-shell is provided to shield surface attached VIP molecules from enzymatic degradation in the airway and inhibits particle aggregation.

At the molar ratio of one VIP per 250 lipid molecules VIP becomes completely associated/incorporated in the liposomes as tested with fluorescent-modified VIP. The concentration of VIP (0.5 mg/ml), however, is high enough, to achieve a therapeutic effect in patients (Petkov et al., 2003).

2.2.1. Extruded liposomes

Some liposomal suspensions were extruded through polycarbonate filters (Millipore, Vienna, Austria) with 100 nm pore size, using a LiposoFast pneumatic extruder (Avestin, Inc. Ottawa, ON, Canada) or, alternatively an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA) in combination with 100 nm polycarbonate filters (Whatman, Kent, UK). Latter was used for nebulisation experiments. According to our experience, 21 extrusion steps are enough to guarantee a homogeneous preparation of large unilamellar vesicles. About 10% of the applied volume is lost during the extrusion procedure. However, we could not detect any release of the electrostatically bound VIP from the liposomes, as verified by fluorescence spectroscopy and HPLC (data not shown).

2.3. Nebuliser

To test the suitability of this liposomal formulation for respiratory application, nebulisation studies were performed using an inhaler with an open-circuit mouthpiece ventilation (MPV).

It is a form of non-invasive ventilation that has been used to provide a portable daytime ventilatory support for patients even with chronic respiratory failure. In clinical medicine, MPV is used because it reduces the risk of respiratory infection due to tracheotomy, and improves cough and voice function as well as patient quality-of-life.

In this study, we used the commercially available inhaler Micro Drop Master Jet (MPV Truma, Putzbrunn, Germany), which produces droplets as small as required for bronchiolar deposition.

2.4. Nebulisation effects

2.4.1. Particle size

Particle size determination was performed by photon correlation spectroscopy (PCS) with a Zetasizer 3000 HSA (Malvern Instruments, Herrenberg, Germany). The particle size was derived by an auto-correlation function, which expresses the averaged liposomal diameter derived from the intensity-weighted size distribution profile. The polydispersity index (PDI) shows the width of the size distribution and is a quality parameter for the homogeneity of the liposomal preparation.

All samples were diluted to a final lipid concentration of 0.03 mg/ml with PBS, which was previously filtered through a disposable 0.02 μm membrane filter unit (Anotop 25, Whatman International Ltd., Maidstone, UK). The measurements were performed at room temperature.

2.4.2. Droplet size

We have determined the median droplet size (DV_{50}) and the droplet size distribution of a nebulised spray mist by laser diffraction using Malvern Spraytec instrument (Malvern Instruments, Herrenberg, Germany). The impact of liposomes on droplet size distribution during nebulisation was addressed. Liposomes were nebulised at room temperature with an atomisation rate of 0.30 ml/min. The spray mist was collected for further examination of liposome size with PCS.

2.4.3. VIP association to liposomes

Fluorescence measurements were performed on a SPEX FLUOROMAX-3 fluorescence spectrophotometer (Jobin Yvon Horiba, Longjumeau Cedex, France) using a 10 mm × 10 mm fluorescence quartz cuvette.

Liposomes loaded with 10 μM Trp-VIP (tryptophan-modified VIP) were selectively excited at 292 nm. The emission wavelengths were assessed between 320 and 380 nm. All data were background corrected by subtraction of the corresponding buffer intensities before determination of the emission intensity maximum.

To address issues of membrane stability and to determine the association behaviour of VIP to liposomes at close to physiological conditions, samples were incubated for 30 min at 37 °C in Krebs–Henseleit buffer (KH buffer) and gassed with oxygen. Nebulised and re-collected Trp-VIP-containing liposomes were examined. Quenching experiments were performed at the same experimental conditions with the water-soluble quencher acrylamide as described previously (Stark et al., 2007). Similarly, we have tested for VIP release during nebulisation using Trp-modified VIP.

2.5. VIP release testing

2.5.1. Artery preparation for relaxation experiments

For the investigation of the release kinetics, we used freshly prepared arteries from young adult male Sprague–Dawley rats weighing 250–280 g (Abteilung für Labortierkunde und Labortiergenetik Medical University of Vienna, Himberg, Austria). We preferred lung arteries to bronchi, because of the well described and excellent dose relaxation response to VIP in the concentration range from 3 to 100 nM (Shahbazian et al., 2007).

The method does not require experimentation on live animals and is well established in our laboratory. Furthermore, this rat model allows for a stringent control of the preparation quality by monitoring the endothelial integrity by response to acetylcholine (Shahbazian et al., 2007). The protocol was according to current national legislation and followed previously published procedures (Petkov et al., 2006). Briefly, under anaesthesia the heart and lungs were excised en bloc, pinned on a wax plate in a Petri dish under cold KH solution. The common pulmonary artery and the left and right extrapulmonary artery segments were opened longitudinally and divided into two pulmonary artery preparations (consisting of common and left or

right pulmonary artery). The preparations were cut as zigzag segments (approximately 13–15 mm), stretched and mounted in thermostatically controlled (37 °C) organ baths. The organ baths contained 9 ml oxygenated KH solution gassed with 95% O_2 and 5% CO_2 (pH 7.4). The activity of the arterial stripes under investigation was recorded isotonically under a resting load of 1 g (Shahbazian et al., 2007). Contraction and relaxation movements were measured by an electronic lever transducer, the signal was amplified by bridge amplifiers (Hugo Sachs Elektronik, March-Hugstetten, Germany) and plotted by ink writers (Watanabe Multicorder MC 6601) with a constant paper speed throughout all experiments. This procedure allows for comparative reaction time measurements and retardation detection.

Reference substances were acetylcholine chloride (ACh) and L-phenylephrine hydrochloride (Phe), and free VIP. Stock solutions of these substances were prepared and further diluted with 0.9% NaCl to the appropriate, final concentrations.

The arterial strips were equilibrated in their organ baths for 2–3 h during which the KH solution was renewed three times. Each artery ran through 4–6 experimental cycles and subsequent washes. One cycle (exemplified in Fig. 2B) consisted of a precontraction by 0.1 μM Phe, a relaxation by the investigational substance (e.g. 100 nM VIP, free or associated with liposomes), followed by the addition of ACh (1 μM), which enables to control that the arterial strips had retained their functionality and vitality during the experimental cycle. Each cycle with the subsequent washes took about 1 h or less.

2.5.2. Experimental design

Each experimental series was accompanied by controls on the same artery from the same animal to minimise bias due to differences between the animals, or differences due to the preparation quality. We tested the acute relaxation power and kinetics of free VIP compared to VLL (extruded and non-extruded). In other experimental series, we tested the release of VIP from liposomes and its biological effect up to 60 min. Then, freshly prepared and liposomes stored for 1–8 months at 4 °C have been compared.

Furthermore, we investigated the reproducibility of VLL production in three batches.

For control purposes we tested empty liposomes, and could not detect any vasoactivity (data not shown).

2.5.3. Data analysis

VIP-induced vasorelaxation was expressed as the percentage of the precontraction recorded immediately before the first addition of VIP. As illustrated in Fig. 2B, the plotted artery relaxation reaction after the substance addition allowed to determine the relaxation power (maximal contraction change) and the time period to achieve the maximal relaxation.

Data in Figs. 4 and 5 are expressed as mean ± S.E.M. for each test candidate. These results contain both, the variance of the artery preparation and the differences between the test candidates. To minimise bias due to differences in the artery preparation, we compared the test candidates with free VIP on the same artery strip. Analyses were performed with the paired Student's *t*-test, comparing two consecutive experiments (control and test formulation) on one artery. Probability values of $p < 0.05$ were regarded as statistically significant.

3. Results

3.1. Liposome size determination before and after nebulisation

The hydration of the dry lipid film resulted in the formation of unilamellar liposomes with a mean hydrodynamic diameter of 175 ± 11 nm (mean ± S.D., for $n = 14$ independent batches) and PDI values between 0.2 and 0.5. A reduction in size and heterogeneity was achieved by size extrusion. Thereafter, liposomes with a mean diameter of 91 ± 3 nm (mean ± S.D., $n = 14$) and PDI values between 0.1 and 0.2 were obtained, irrespective of whether VIP was present or not ($p > 0.5$).

To follow the impact of nebulisation on liposomal particle size distribution the spray mist was collected and analysed. For these batches of liposomes the hand extruder was used resulting in a mean particle diameter of 104 ± 3 nm ($n = 3$) before nebulisation. After nebulisation the particle size was determined as 110 ± 5.1 nm ($n = 3$). Thus, neither the particle size nor the PDI values (0.1–0.2) were significantly increased ($p > 0.3$) after nebulisation.

When nebulised liposomes (loaded with Trp-VIP) were subjected to fluorescence spectroscopy and acrylamide quenching, we did not detect any release of Trp-VIP.

The spectra for nebulised VLL were indistinguishable from those of non-nebulised samples (data not shown). Accordingly, a similar low quenching constant was determined for nebulised VLL before (Stark et al., 2007). The results did not indicate any evidence for vesicle disruption and/or VIP release due to nebulisation.

3.2. Spray mist characterisation

The volume median diameter (DV_{50}) of particles in the spray mist produced by the MPV inhaler was determined as ~6 μm, irrespective of whether liposomes in PBS or pure PBS buffer without liposomes were atomised (Fig. 1A).

The spray pattern in terms of volume frequency (%) shows the droplet size distribution ranging from 1 to 20 μm with 90% of the droplets being below 14 μm in size (Fig. 1B).

3.3. Biological VLL testing

3.3.1. Relaxation pattern

Fig. 2 shows a plot with curves derived from free VIP and from VLL. The curves allow to (1) measure the extent of precontraction, (2) determine the maximal relaxation (relative to the precontraction), and (3) assess the time from substance addition to maximal relaxation.

3.3.2. Acute relaxation by extruded and non-extruded liposomes

Fig. 3 summarises the results for the relaxation behaviour of different VIP formulations. Both, free VIP and VLL have

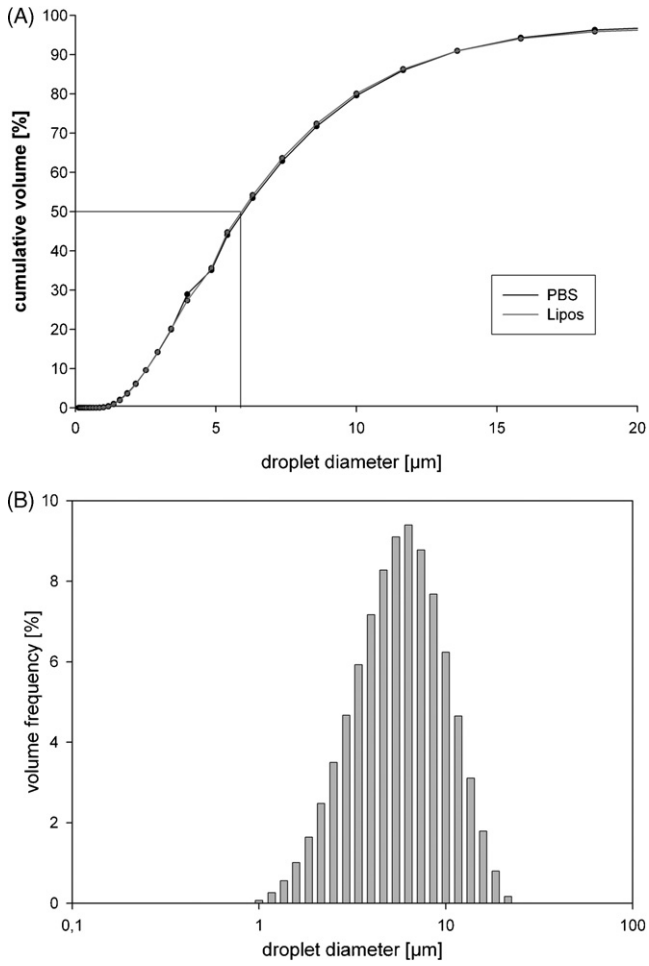

Fig. 1. Aerosol droplet size distribution. Following the MPV inhaler dispersion, the droplet size distribution was determined with laser diffraction (A). The volume median diameter (DV_{50}) was about 6 μm for both, pure PBS droplets and liposomal enriched droplets (B). An analysis of the spray pattern distribution in terms of the corresponding volume frequency (%) shows more than 90% of the droplets being below 14 μm in diameter.

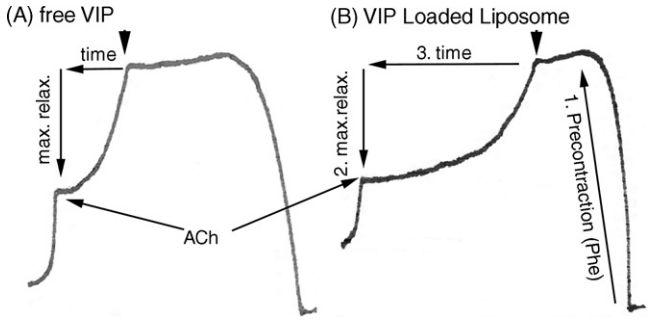

Fig. 2. Smooth artery relaxation movement. Rat artery *ex vivo*, plotted vasorelaxation curve; the arteries were stimulated with phenylephrine (Phe) to reach 100% precontraction. Treatment (↓) was with 100 nM free VIP (A) or equimolar VIP in VLL (B). The treatment is followed by further relaxation with acetylcholine (Ach) to show the viability and responsiveness of the artery. Note, that the relaxation movement in (B) is smooth, indicating a continuous substance release from the VLL, rather than a breakdown-associated sudden release. The plots served as basis for the analysis of maximal relaxation and time to the peak relaxation as illustrated in (B) relative to 100% (1, precontration), we measured the contraction change (2, max. relaxation), and the reaction duration (3, time).

the potential to relax the arteries, which is experimentally observed in a reduction of precontraction. For free VIP, the relaxation expressed as percent of the precontraction was $38.53 \pm 2.34\%$ (mean ± S.E.M.; $n = 48$). For extruded VLL and non-extruded VLL the relaxation was $30.35 \pm 3.12\%$ ($n = 36$) and $29.52 \pm 2.60\%$ ($n = 41$), respectively. The difference between free VIP and each liposomal formulation was significant ($p < 0.05$) by means of the paired Student's *t*-test, while extruded and non-extruded VLL were not significantly different from each other ($p > 0.1$).

Fig. 3B summarises the time period from substance addition to maximal relaxation. For free VIP, the time to achieve maximal relaxation was 282 ± 11 s (mean ± S.E.M.,

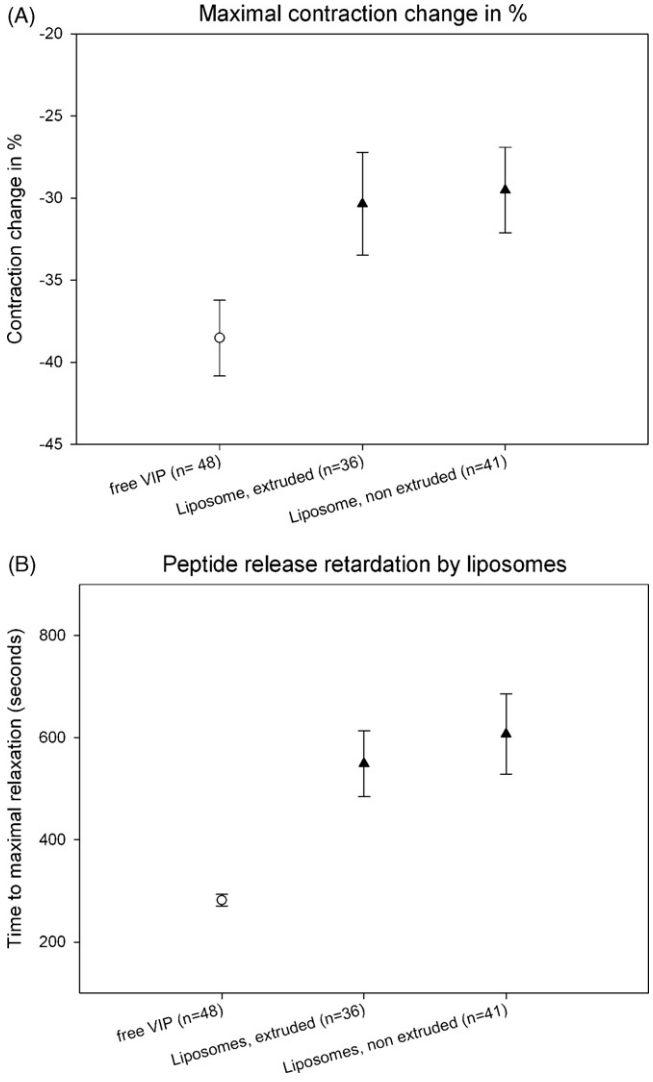

Fig. 3. Substance retention by liposomes. Relaxation efficacy of 100 nM VIP, added to the life artery *in vitro* either as free VIP, or associated with extruded or non-extruded liposomes. (A) Extruded and non-extruded VLL reveal a depot effect as indicated by a reduction of the relaxation. Both liposomal preparations reveal a similar peak relaxation (contraction change). (B) Time to the peak relaxation of free VIP, or equimolar VIP associated with extruded, or non-extruded liposomes. Compared to free VIP, both liposomal preparations significantly extend the time period from substance addition to the peak relaxation. Extruded and non-extruded liposomes reveal a similar retardation.

$n = 48$). The time period was highly significantly ($p < 0.0001$) extended to 549 ± 64 s ($n = 48$) and 607 ± 78 s ($n = 36$) for equimolar amounts of VIP-loaded extruded or non-extruded liposomes, respectively. Extruded and non-extruded liposomes revealed no significant difference ($p > 0.1$) in the time period to reach the maximal relaxation (Fig. 3B). From fluorescence spectroscopy using fluorescent-modified VIP we got no experimental evidence that liposome-associated VIP is unspecifically released from the liposomes simply by incubation with the organ bath without arteries (data not shown).

3.3.3. Sustained VIP release

In an extra set of experiments, we observed the acute relaxation response, and in addition the relaxation status up to 1 h after substance addition (Fig. 4). Addition of free VIP was followed by an acute relaxation after about 4 min and a rapid re-contraction, which was completed within 60 min. In contrast, the VLL slightly reduced the acute VIP-mediated relaxation but inhibited the rapid re-contraction. After 60 min a significant relaxation due to sustained VIP release from the VLL was observed. Due to the VLL-mediated re-contraction delay, after 10 min and later the relaxation was more than with free VIP (Fig. 4).

3.3.4. Batch reproducibility

We have tested three different batches of extruded unilamellar VLL to survey the reproducibility of liposomal preparations. When compared to equimolar amounts of free VIP, all liposomal batches consistently revealed a similar reduction of relaxation power combined with an almost identical extension time to the maximal relaxation (data not shown).

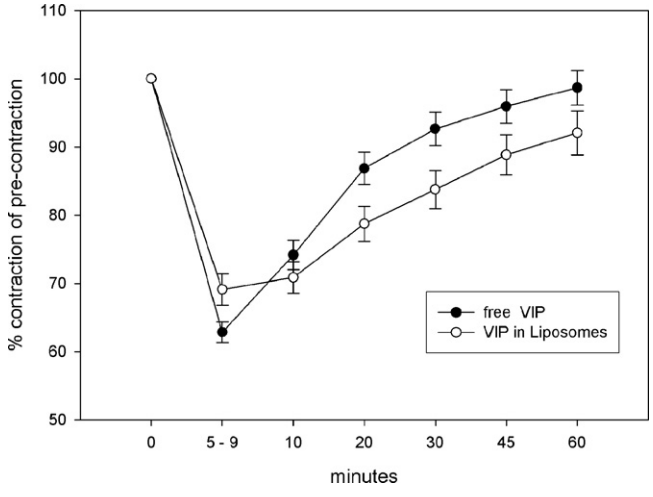

Fig. 4. Sustained activity by slow release. Relaxation efficacy of 100 nM VIP, added to the life artery *in vitro* either as free VIP, or VLL. Free VIP reduced the contraction immediately from 100% to below 70%, following the maximal relaxation the arteries re-contracted completely after 1 h. With VLL, the initial relaxation was weaker. However, already 10 min after substance addition and relaxation onset VIP in VLL produced more relaxation than free VIP. In contrast to free VIP – due to the sustained VIP release – VLL produced significant relaxation after 60 min.

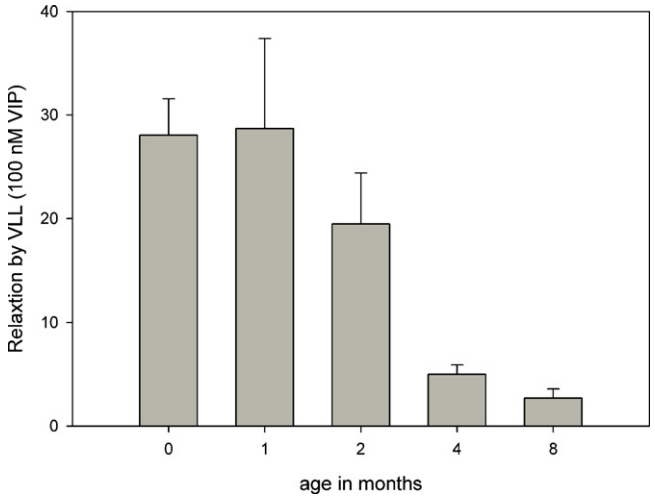

Fig. 5. Relaxation efficacy of fresh and stored liposomes. Freshly prepared and stored VLL were compared for their ability to relax rat arteries. Fresh VLL ($n = 48$) and stored for 1 month at 4 °C showed similar activity; though reduced, it was still sufficient after 2 months, after 4 months ($n = 37$) only a minor relaxation response was observable, which was further reduced when the storage period was 8 months.

3.3.5. Biological effect of stored liposomes

One could imagine that VLL exhibit a loss in their biological activity during storage. Therefore, we have tested freshly prepared as well as stored liposomal formulations. The results are shown in Fig. 5. We found that the relaxation activity of VLL remained intact for at least 1 month. After 2 months the relaxation activity was impaired, and after 4–8 months of storage, hardly any biological activity could be detected.

4. Discussion

Because pulmonary delivery of exogenous VIP is a promising administration route for the treatment of lung diseases, we have developed a delivery system intended for inhalation of VIP (Stark et al., 2007). In this study, we have investigated the stability and integrity of VLL after nebulisation with a commercially available inhaler. The MPV inhaler was chosen as it produces a homogeneous droplet size distribution with 50% of the droplets being smaller than 6 μm, which is a size suited to reach the lower bronchioles (Labiris and Dolovich, 2003).

Finally, by using VIP-mediated vasorelaxation *in vitro*, we observed favourable VIP-release kinetics, suggesting a high potential to improve the envisioned VIP inhalation therapy.

4.1. Nebulisation effects

We could show that the liposomes remain stable after nebulisation, as we found no statistically significant change in size indicative for liposomal degradation or aggregation (Fig. 1). As it is known that high shear forces produced during nebulisation might induce liposomal fragmentation dependent upon parameters as liposomal composition or size (Niven and Schreier, 1990), this finding further supports the suitability of our liposomal formulation for inhalation. Regarding liposomal size, Niven et al. (1991) reported that the leakage of drugs is minimised

for smaller liposomes, which are progressively smaller than the aerosol droplet. This is clearly the case for VLL, moreover, in VLL the VIP molecules are tightly associated to the lipid membrane being additionally protected by the PEG-shell. The PEG-shell might also increase the stability and integrity of the liposomes by inhibiting particle aggregation during nebulisation. Finally, PEGylation might be of advantage for the preservation of membrane integrity in the environment of epithelial lining fluid in the lungs (Anabousi et al., 2006).

Because nebulisation by the MPV inhaler did not result in any detectable VIP release, we conclude that VLL can be inhaled without prior release of VIP due to the inhalation process itself. However, as the operation conditions (Niven et al., 1992) as well as the utilized inhaler device strongly influence the physical stability of liposomes, the results presented here are only strictly valid for the MPV inhaler used within this study.

4.2. Vasorelaxation potential and release from unilamellar liposomes

For the assessment of the relaxation potential of liposomal-associated VIP and the release kinetics out of the VLL, we have chosen an *ex vivo* method, which is commonly used in pharmaceutical sciences for measuring the efficacy of vasorelaxing substances. We have used strips of arteries instead of bronchi because arteries contain more muscle cells with VIP receptors.

In all experiments, we have administered 100 nM VIP, a pharmacologic concentration just below receptor saturation (Shahbazian et al., 2007). In that case, the relaxation curve after administration of a single dose directly reflects the amount of available and active VIP, and a continuous recording allows for the estimate of bioactive VIP available to the cells of the artery at any given time point (Figs. 2–4).

4.2.1. Liposomes allow for a smooth peptide release

In the relaxation movement (Fig. 2) we found no indication for a sudden breakdown of the liposomes or a discontinuous release of VIP. In contrast, there was a smooth vasorelaxation response towards effect saturation, indicating that our liposomal formulation allows for a continuous and smooth peptide release as seen in Fig. 2B.

4.2.2. Liposomes retard relaxation

The maximal vasorelaxation observed with 100 nM free VIP was close to 40% relaxation (relative to 100% precontraction). Both liposomal formulations (extruded and non-extruded) revealed a statistically significant lower maximum relaxation compared to free VIP (Fig. 3A), indicating that the smooth release is retarded.

For the relaxing efficacy and hence the substance release, it was of little relevance of whether the liposomes were extruded or not (Fig. 3). Thus, the question if one has to perform size-extrusion before nebulisation is irrelevant for our liposomal formulation and its function. However, it might be important from the pharmatechnological point of view in terms of batch-to-batch standardization. In our study, three independent batches of liposomes showed similar peptide retention, arterial relaxation and release retardation, emphasizing an adequate reproducibility in the manufacturing protocol for the unilamellar VLL.

As the relaxation response was observed for longer, i.e. up to 60 min, the VLL were clearly superior in the vasorelaxation response as compared to free VIP (Fig. 4), most likely due to a sustained release. This finding may classify the liposomal drug delivery system as a depot formulation for VIP. Consequently, it can be inferred that the retarded release of active VIP from VLL could significantly improve the relaxation efficacy of VIP.

In view of the intended VLL inhalation it might be interesting that we observed no "sudden breakdown of the liposomes" (Fig. 2). Because our biological model was in "close to physiologic" buffer, it is likely that the same release kinetics in practice (if VLL are deposited on the walls of the airways lined with mucus or surfactant) contribute to a sustained VIP delivery to the target cells.

Our relaxation experiments indicate a loss of VIP bioactivity after some storage time. We do not know whether the peptide function loss after 2 months (Fig. 5) is attributable to peptide denaturation, or degradation, or possible interference with a liposome component. Therefore, appropriate storage conditions have to be established.

4.3. Release is triggered by the target

We tested for the retention of fluorescent-modified VIP in KH buffer, at 37 °C, gassed with O_2, simulating the environmental conditions of the organ bath without arteries. Concerning liposomal stability during storage we already know that the VLL are stable in PBS without any loss of their peptide load (Stark et al., 2007). When delivered to target cells in the tissue, however, we observed an immediate relaxation onset (Fig. 2). Consequently, because the VLL did not show any release in solution without target cells, we assume that the ligand–receptor interaction triggers the release of VIP from the liposomes. Most probably, the VIP molecules embedded in the outer monolayer of the liposomes are released earlier than the VIP molecules entrapped in the interior of the nano-carrier. Likewise, the alpha helical structure of VIP in VLL supports efficient receptor uptake (Sethi et al., 2005). In summary, our findings suggest that VIP-molecules are released in the alpha helical conformation from the VLL by direct contact with the receptor.

4.4. Relevance for other peptides

The generation of liposomal peptide carriers requires some specific tailoring of the liposomal components to the peptide chemistry and biology. As other peptides share parameters with VIP, it seems reasonable that the specific liposomal formulation in combination with the manufacturing technique developed for VIP can be adapted to other peptide drugs.

Accordingly, peptide-loaded nano-formulated liposomes offer the opportunity to increase peptide availability by (i) protection from degradation by macrophages and peptidases in the airways, and, as shown here and (ii) by prolongation of the spe-

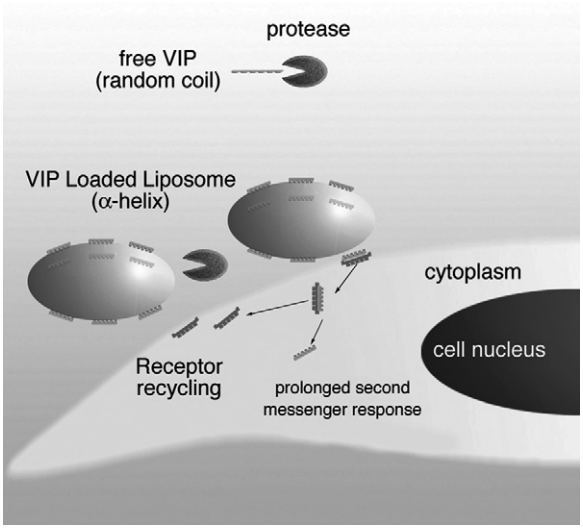

Fig. 6. Suggested function model of VLL. Free VIP (random coiled) can easily be degraded by proteases on the way to the receptor. VIP from VLL is protected against proteases. VLL show no peptide leakage during storage, but electrostatically bound VIP may become released by direct contact and binding to the receptor. Following the ligand–receptor-complex internalisation, and the intracytoplasmic complex disintegration, the receptor is recycled to the cell surface to bind new upcoming VIP molecules. In addition to the protection by liposomes *per se*, the alpha helical conformation of VIP induced by negatively charged liposomes may convey further degradation protection; moreover, it is the preferred VIP-conformation for receptor binding.

cific activity indicating a sustained substance release in the lung (Figs. 3 and 4).

4.5. Conclusion

We suggest a synergy effect from recycled receptors and sustained release from VLL improving the biological and pharmacological activity of VIP. In a hypothetical scheme shown in Fig. 6 we illustrate that both, protection and release retardation can increase the chance for a VIP molecule to survive enzymatic degradation and to find a recycled receptor on the cell surface.

As shown for systemic applications (Önyüksel et al., 2006), the retarded release of VIP from VLL extends the effective half-life and increases the therapeutic efficacy of VIP. Our data indicate that VLL can be nebulised for inhalation and therefore allow for the administration of an intra-bronchial depot system. These findings warrant further research and preclinical toxicology investigations of the VIP-loaded liposomes before clinical trials can be performed.

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