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Mechanisms of a phosphorothioate oligonucleotide delivery by skin electroporation

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Abstract

Skin electroporation has great potential for topical delivery of oligonucleotides. Controlled therapeutic levels of an intact phosphorothioate oligonucleotide (PS) can be reached in the viable tissue of the skin. The aim of this work was to investigate the transport mechanisms of a PS in hairless rat skin by electroporation, and hence to allow optimization of oligonucleotides (ONs) topical delivery. The pulsing condition used was five exponentially-decaying pulses of 100 V and 500 ms pulse time. The main mechanism of PS transport in the skin viable tissues during pulsing was electrophoresis. The electroosmosis contribution was negligible. Electrophoresis created within minutes a reservoir of PS in the skin viable tissues, which persisted within a therapeutic range for hours. A strong PS/stratum corneum interaction occured. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Electroporation; Mechanisms; Oligonucleotides; Skin; Topical delivery; Transdermal delivery

1. Introduction

Electroporation is a phenomenon in which lipid bilayers exposed to high intensity electric field pulses are temporarily destabilized and permeabilized (Weaver, 1995). Although the molecular mechanism by which electric fields interact with lipid bilayers is still incompletely understood, it is generally accepted that electric pulses create transient aqueous pathways within the membrane bilayers. The most common use of high voltage pulsing is the introduction of DNA into isolated cells. Hence, it has been used for introducing oligonucleotides (ONs) into cells (Bazile et al., 1989; Klenchin et al., 1991; Bergan et al., 1993). Recently, high voltage pulsing has been shown to enhance the transdermal transport of different molecules. Increases in molecular transport between one and four orders of magnitudes have been achieved, depending on the size, the electric charge or the solubility of the molecule to be transported, and the electric protocol used (Prausnitz et al., 1993a; Jadoul, 1997; Vanbever et al., 1996a; Vanbever, 1997) .

Antisense ONs have been proposed as new therapeutic agents by their ability to selectively interfere with gene expression. Research in this fast growing field has led some antisense compounds to advanced clinical trials (Crooke and

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Bennett, 1996). However, due to their physical properties, ONs are not expected to be orally active. Moreover, once in the general circulation, they are widely distributed and may be degraded before reaching the target cells. They have to cross the plasma membrane and reach their molecular target, with which they must interact specifically. Many methods have been proposed to increase ON delivery to the target cells (e.g. by encapsulation in immunoliposomes, coupling to ligands or topical delivery), and to increase their resistance to nucleases and/or cell penetration (e.g. by chemical modifications or association with drug carriers; see Rojanasakul, 1996 for a recent review).

Due to its accessibility, the skin is a privileged tissue for topical delivery. In addition, many cutaneous diseases (e.g. skin carcinoma, melanoma, psoriasis, viral diseases such Herpes Simplex Virus-1) are potential candidates for antisense therapy. However, transdermal transport of macromolecules is impeded by the barrier properties of human skin, mainly due to the stratum corneum. The application of highly concentrated methylphosphonate solutions for hours allowed however to achieve therapeutic levels of methylphosphonate in the skin viable tissues (Nolen et al., 1994). To increase the transdermal transport of ONs, iontophoresis (Vlassov et al., 1994; Oldenburgh et al., 1995; Brand and Iversen, 1996) has been successfully used. Electroporation, using numerous short duration pulses (Zewert et al., 1995) was less efficient. Pulsing with low number of medium voltage–long duration pulses achieved therapeutic levels of phosphorothioate ON in the skin viable tissues and allowed a control of the quantities delivered to the skin. The phosphorothioate ONs were stable for at least 4 h in the viable skin (Regnier et al., 1998). Transdermal electroporation has also been reported to permeabilize cells in vivo, as shown by electrochemotherapy, (i.e. the local potentialization of bleomycin effect by electric pulses; Belehradek et al., 1994), or efficient transfection of mouse skin cells by plasmid vectors (Titomirov et al., 1991; Zhang et al., 1996). Our working hypothesis is that electroporation increases ON permeation through permeabilized stratum corneum, and promotes permeation of ONs in the epidermal cells (Regnier et al., 1996, 1998).

This aim if this work was to investigate the transport mechanisms of a model ON in the skin viable tissues by high voltage pulsing and hence to allow optimization of topical delivery of ONs.

2. Materials and methods

2.1. Oligonucleotides

A phosphorothioate oligodeoxynucleotide (PS, 5′ ACC AAT CAG ACA CCA 3′, 4740 Da, $z = -14$; Eurogentec, Seraing, Belgium) was used as a model oligonucleotide given that it resists for hours to degradation by skin nucleases (Regnier et al., 1998). It is complementary to a site of the UL 52 essential gene of Herpes Simplex Virus-1 (Roizman, 1992). PS was purified by preparative Polyacrilamide gel electrophoresis (PAGE; Regnier et al., 1998). PS was labeled at the C8 positions of the purine bases by hydrogen exchange with tritiated water (Regnier et al., 1998), according to the method of Graham et al. (1993). The specific activity of the ^{3}H-labeled PS was 0.11 mCi/μmoles. Purity of the ^{3}H-labeled PS was checked by PAGE.

2.2. In vitro delivery of PS

The in vitro model is a polycarbonate vertical diffusion chamber made of two compartments separated by hairless rat skin, with epidermis facing the upper (donor) compartment. Full-thickness abdominal rat skin samples (Mutant Iops hairless rat, Iffa Credo, St Germain les Arbresles, France) were prepared by gently scraping off subcutaneous fat of freshly excised skin. The skin area exposed to both solutions was 1 cm^2. Platinum pure electrodes (0.25 cm^2, Johnson Matthey, Brussels, Belgium) separated by 1 cm were set in both upper and lower compartments. Unless otherwise mentioned, the cathode was in the upper compartment while the anode was in the lower compartment. Unless otherwise noted, the upper and lower compartment were filled with 0.5 ml of ^{3}H-labeled PS solution (3.3 μM ^{3}H-PS in 0.04 M

Hepes buffer, pH 7) and 2.5 ml of 0.024 M phosphate buffer, pH 7.4 isotonized with 4% w/v glucose, respectively. No shift in pH in the donor nor receptor solutions due to pulsing or iontophoresis was observed. The lower compartment was maintained at 37°C and continuously stirred.

PS permeation was measured at various times after electric treatments, as mentioned in the text and in the figure captions. Unless otherwise noted, PS was allowed to diffuse for 5 min before pulsing. Results were compared to unpulsed passive diffusion controls.

2.3. Electric protocols

The electroporation device used is an Easyject Plus® (Cell One, Herstal, Belgium), delivering exponentially-decaying (ED) capacitive discharge pulses, characterized by their initial voltage ($U_{electrodes}$) and their pulse time (τ). The time required for the voltage to drop to 37% of its initial value corresponds to τ. Unless otherwise noted, the pulsing protocol used consisted in applying five pulses of 100 V and $\tau = 536$ ms ($5 \times$ (100 V—536 ms)). Pulses were separated by 1 min. Previous results have shown that a therapeutic level of PS can be reached in the skin viable tissues after PS delivery by this pulses series (Regnier et al., 1998).

Iontophoresis (0.3 mA/cm^2) was applied for 13 min using a constant-current power supply. The total charge transferred ($Q_{ionto} = 0.23$ C), calculated following Ohm's law was chosen to correspond to the total charge transferred by $5 \times$ (100 V—457 ms) pulses. $Q_{[5 \times (100\ V—457\ ms)]}$ (0.22 C) was calculated from the equation $Q = n \cdot \tau \cdot (U_{electrodes})/R_{electrodes}$, where n is the number of pulses, τ is the pulse time, $U_{electrodes}$ is the initial applied voltage and $R_{electrodes}$ is the resistance of the diffusion chamber when $U_{electrodes}$ is maximum (Vanbever and Préat, 1995). $R_{electrodes}$ was calculated according to Ohm's law, by monitoring $U_{electrodes}$ by a digital oscilloscope (HP54601A, Hewlett Packard) and the initial current across a series resistance. Skin resistance when $U_{electrodes}$ is maximum (R_{skin}), was calculated by subtraction of the resistance of the donor and receptor solutions from $R_{electrodes}$. We evaluated U_{skin} from the formula $U_{skin} = U_{electrodes} \cdot R_{skin}/R_{electrodes}$. PS was allowed to diffuse passively for 9 min before pulsing, to standardize the total time of exposure of PS to the skin.

2.4. Quantification of PS in the stratum corneum and in the skin layers

After pulsing, PS was allowed to diffuse during various times (post-pulsing diffusions). Then, the skin was recovered from the diffusion chamber and was gently wiped clean. The area exposed to the donor solution was excised, pinned flat and tape-stripped nine times (Scotch Cristal, Cergy Pontoise, France) to remove the stratum corneum. Each strip and the remaining skin tissues (which we called skin viable tissues, corresponding to the viable epidermis and dermis) were freeze-dried to remove the tritium label which might have exchanged with water. The dry samples were digested over 24 h with 1 ml of 1 N NaOH, then neutralized with 167 μl of 6 N HCl and mixed with liquid scintillation cocktail (Ready Safe, Beckman, Belgium). Finally, each vial was counted for radioactivity (Beta Counter, Wallac 1410, LKB, Pharmacia). ON quantities were calculated from the total ^{3}H radioactivity in the stratum corneum or the viable tissues of the skin. They are expressed as mean $\pm$ standard error of the mean ($n = 3$–8) and were compared by two ways analysis of variance (Anova, $p < 0.05$). The PS concentration in the stratum corneum or in the skin viable tissues was calculated by dividing the quantity of ON by the corresponding tissue volume (1.4 μl for the stratum corneum and 65 μl for the viable tissues; Regnier et al., 1998).

3. Results

3.1. PS transport during pulsing

As for iontophoretic delivery, the net transport of the oligomer during pulsing could result from electrophoresis, electroosmosis and/or passive diffusion. The electroosmotic flow (i.e. the convective water flow) is from the anode to the cathode, due to the skin's cation permselectivity (Guy,

1992), whereas electrophoresis drives anionic molecules from the cathode towards the anode.

To evaluate the transport of PS during pulsing, the quantity of ON that penetrated the skin tissues was determined directly after the electric treatment. To investigate the implication of electrophoresis on the net transport of PS during forward pulsing, pulses were applied with competitive ions added to the donor solution. Further, we compared forward and backward pulsing (with the cathode or the anode in the donor compartment, respectively).

Forward pulsing strongly promoted the transport of PS in the skin viable tissues, compared to the 10 min unpulsed diffusion control (Fig. 1A). In contrast, backward pulsing did not increase PS transport compared to the control. This shows

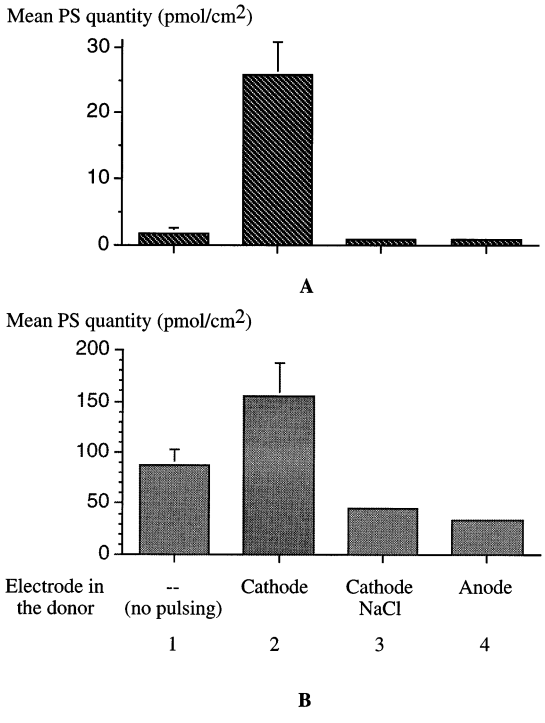

Fig. 1. PS transport during pulsing. Mean quantity of PS (pmol/cm^2) recovered in the skin viable tissues (A) or the stratum corneum (B) directly after pulsing (5 × (100 V, $\tau \approx 500$ ms)) with the cathode (2) or the anode (4) in the donor compartment. The donor compartment was filled with 3.3 μM PS in a 0.04-M Hepes buffer, pH 7 (2, 4), or with the 3.3 μM PS + 0.15 M NaCl in the same buffer (3). The results were compared with a 5-min passive diffusion control (1).

that electrophoresis was more efficient than electroosmosis to drive PS into the skin viable tissues. Moreover, forward pulsing with 0.15 M NaCl in the donor compartment decreased the transport of PS to a value not significantly different from the unpulsed passive diffusion control, confirming that PS electrophoresis is the main mechanism of PS permeation during pulsing.

The quantity of PS was markedly more elevated in the stratum corneum than in the viable tissues of the skin, showing that, although electropermeabilized, the stratum corneum was still an efficient barrier to permeation (Fig. 1A vs Fig. 1B). After 10 min passive diffusion, 90 pmoles of PS were recovered in the stratum corneum (Fig. 1B), which correspond to a concentration of 64.3 μM, vs 3.3 μM in the donor compartment. PS thus accumulated in the stratum corneum independently of pulsing. Finally, whereas forward pulsing did not significantly promote the accumulation of PS in the stratum corneum, forward pulsing in the presence of NaCl and backward pulsing decreased the accumulation of the oligomer in this layer. This suggests that, during pulsing, electrophoresis was less involved in the increase of the quantity of PS in the stratum corneum than passive transport. However, the electric pulses could electroelute part of PS that passively associated with the dead tissue when the polarities were reversed. The effect of NaCl could be linked to the ionic nature of PS interaction with the stratum corneum (reduction of PS affinity for the stratum corneum) and/or to competition with PS for electrotransport.

3.2. PS transport after pulsing

3.2.1. PS absent during pulsing

The second step was to investigate the long lasting effects of electroporation on the penetration of PS in the skin after pulsing. Previous studies showed significant transport of small molecules after pulsing (Vanbever et al., 1997a). However, it has to be determined whether the permeabilized structures allow the passage of medium-sized polyanions like ONs through the stratum corneum after pulsing. Therefore, we pulsed hairless rat skin with a PS-free Hepes

buffer in the donor compartment, and 1, 15 or 60 min after pulsing, we replaced it with the PS solution. PS was then allowed to diffuse for 4 h. Results were compared with an unpulsed 4 h passive diffusion control.

The transport of PS in both the skin viable tissues (Fig. 2A) and the stratum corneum (Fig. 2B) increased when the electric pulses were applied to the tissue before setting the oligomer in the donor compartment. However, the post-pulsing transport of PS decreased as the compound was added later after pulsing. This demonstrates that the permeability of the stratum corneum to PS remained increased for at least 1 h.

When the pulses are applied in the absence of PS, the transport of the drug after the electric treatment and 4 h diffusion is equivalent to the transport during pulsing (5 min; Fig. 2A vs Fig. 1A).

3.2.2. PS present during pulsing

To determine whether PS can significantly be transported in the target tissues after the electric protocol when pulses are applied in presence of the oligomer, we compared the quantities of PS found in the skin just after pulsing or 4 h after pulsing in the presence of PS.

When pulses were applied in the presence of the PS, the quantities of drug recovered in the skin viable tissues just after pulsing or 4 h after pulsing were not different (Fig. 3A). In contrast, the quantity of PS found in the stratum corneum increased when PS was allowed to diffuse after pulses (Fig. 3B). Similar results were obtained with pulses of 200 V (data not shown).

To evaluate the release of PS from the reservoir created within the skin during the electric treatment, we compared the quantity of PS found in both the stratum corneum and the skin viable tissues just after pulsing with the quantity of PS found in these tissues 4 h after the replacement of the donor solution by a PS-free buffer.

Equivalent results were obtained when replacing the solution of PS by a PS-free buffer after pulsing, in both the skin viable tissues (Fig. 3A) and the stratum corneum (Fig. 3B). This shows that PS did not move within the skin after pulsing. Furthermore, PS did not diffuse in the receptor compartment after pulsing given that the transdermal permeation of the drug was very low (only a few pmol/cm^2 after 4 h, data not shown). Therefore, the reservoir of PS created during pulsing in the stratum corneum and in the viable tissues lasted more than 4 h.

The last step was to demonstrate the low diffusion of PS after the electric treatment when the pulses were applied in presence of the oligonucleotide. For that purpose, the [^{3}H] PS solution was placed in the donor compartment only after the pulses, pulsing being performed in presence of unlabeled PS. The radiolabeled PS was then allowed to diffuse for 4 h. This allowed us to measure only the post-pulsing diffusion of PS when pulsing in presence of the oligonucleotide.

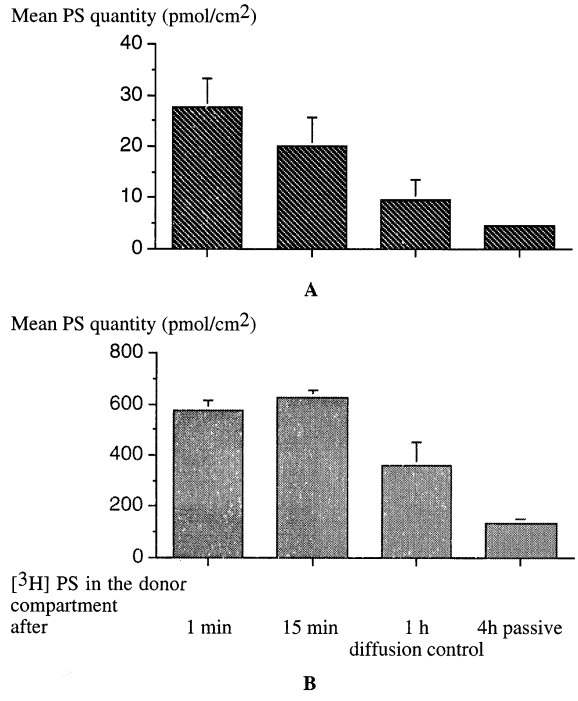

Fig. 2. Post-pulsing transport of PS, PS being absent during pulsing. Mean quantitiy of PS (pmol/cm^2) in the skin viable tissues (A) or in the stratum corneum (B). During pulsing, the donor compartment was filled with 0.5 ml 0.04 M Hepes buffer, pH 7. One, 15 or 60 min after pulsing, the donor compartment was emptied and filled with 3.3 μM Hepes buffer, pH 7. PS was allowed to permeate the skin for 4 h. Results were compared with 4 h passive diffusion control.

Pulsing in presence of unlabeled PS hindered [^{3}H] PS transport in the skin viable tissues after pulsing ceased (Fig. 3A). This was not observed in the stratum corneum (Fig. 3B) nor in the viable tissue when pulsing was performed in the absence of PS.

3.3. Iontophoresis vs electroporation

As electrophoresis was mainly responsible for PS transport across electropermeabilized stratum corneum, the total charge transferred (Q) was chosen as a basis of comparison between the two methods of drug delivery. Both electroporation and iontophoresis conditions were chosen to transfer a total charge of approximately 0.22 C. The quantities of PS in the skin were determined just after the electric treatment to avoid artefacts due to diffusion.

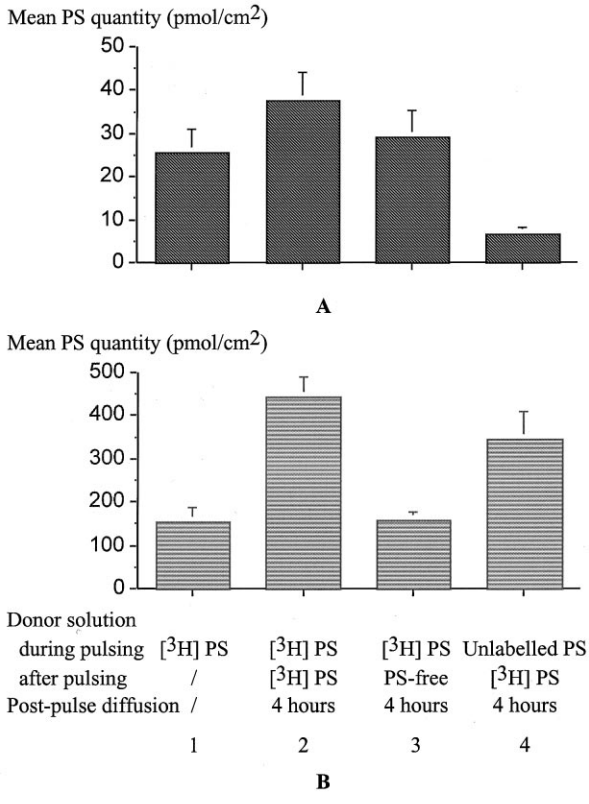

Fig. 3. Post-pulsing transport of PS, PS being present in the donor compartment during pulsing. Mean quantity of PS (pmol/cm^2) recovered in the skin viable tissues (A) or the stratum corneum (B) 4 h after pulsing ($5 \times (100$ V, $\tau \approx 500$ ms)). 1, 2, 3: The donor compartment contained [^{3}H] PS during pulsing. The quantity of oligomer was determined just after pulsing (1), 4 h after pulsing (2) or 4 h after replacement of the [^{3}H] PS solution with a PS-free Hepes buffer 1 min after pulsing (3). 4: Pulsing was performed with unlabelled PS in Hepes buffer in the donor compartment. One minute after pulsing, the donor solution was discarded and replaced with 0.04 M Hepes buffer, pH 7.

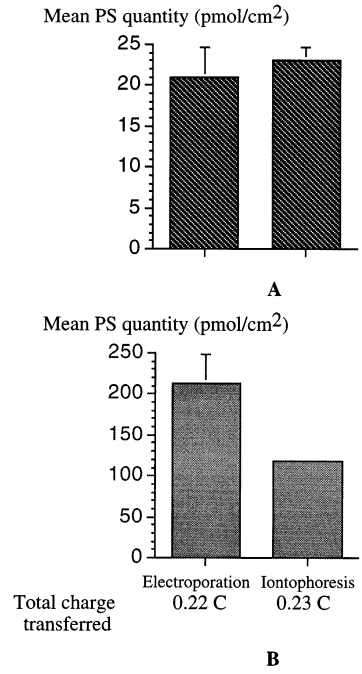

Fig. 4. Iontophoresis vs electroporation. Mean quantity of PS (pmol/cm^2) recovered in the skin viable tissues (A) or the stratum corneum (B) directly after pulsing ($5 \times (100$ V, $\tau \approx 450$ ms)) or after 13 min iontophoresis. Donor solution: 3.3 μM PS in 0.04 M Hepes buffer, pH 7.

Fig. 4A shows that PS was delivered in the skin viable tissues in equivalent quantities by both delivery methods. In contrast, iontophoresis was less efficient than electroporation to drive the molecule in the stratum corneum (Fig. 4B). Moreover, the quantity of PS measured in this layer after iontophoresis or passive diffusion was not significantly different (data not shown).

4. Discussion

In this study we investigated the mechanisms of transport by electroporation of PS, a medium

sized antisense oligonucleotide (4690 Da, $z = -14$). Since we aimed at topical delivery, we were mainly interested in the transport of the molecule in the skin tissues, especially the skin viable tissues (viable epidermis and dermis), which are potential targets for antisense therapy (Greenhalgh et al., 1994; Regnier et al., 1998).

Electric pulses have been shown to promote transdermal delivery of various molecules, by: (i) rapid and prolonged permeabilization of the skin, allowing significant enhancement of molecular transport by passive diffusion after or between pulses (Prausnitz et al., 1993b; Vanbever et al., 1996b, 1997a); and (ii) electrophoresis of charged species through the permeabilized structures (Prausnitz et al., 1993b; Vanbever et al., 1994). The relative contribution of electrophoresis over enhanced diffusion could be dependent upon the physicochemical properties of the molecule as well as the donor solution (Pliquett and Weaver, 1996; Vanbever et al., 1996b, 1997a). The contribution of electroosmosis (i.e. the convective flow of water towards the cathode, due to the skin's cation permselectivity (Guy, 1992)) has been shown to be negligible (Vanbever et al., 1996b).

4.1. Dependence of PS transport upon electrophoresis

The main mechanism of transport of PS in the skin viable tissues during pulsing is electrophoresis (Figs. 1 and 3), the contribution of electroosmosis on PS transport being negligible, as previously reported for other molecules (Vanbever et al., 1996b). This explains the dependence of the quantity of PS delivered to the skin by electroporation upon the total amount of charges transferred (Regnier et al., 1998). This strong involvement of electrophoresis in the mechanisms of transport by electric pulses was also reported by Zewert et al. (1995) for transdermal delivery of a 25-mer phosphorothioate oligonucleotide and by Klenchin et al. (1991) for DNA uptake by cells.

4.2. Interaction of PS with the stratum corneum

When PS is placed in the donor compartment after pulses, the post-pulse diffusion is significantly increased after more than 1 h compared to the unpulsed control (Fig. 2). The increase in PS diffusion after pulsing indicates that structural changes occurred in the stratum corneum, promoting PS diffusion. However, pulsing in the presence of PS prevents the penetration of the oligomer in the skin viable tissues after pulsing (self-decrease of the post-pulsing transport of PS; Fig. 3). In contrast, the presence of PS during pulsing does not modify the quantity of PS detected in the stratum corneum (Fig. 2 vs Fig. 3). As proposed by Prausnitz et al. (1995), the molecules transported during pulsing may interact with the pathways, leading, in the case of heparin, to a self-enhancement of the transdermal flux of the molecule. Likewise, PS might interact with the pathways, but the interaction would lead in this case to a self-decrease of the post-pulsing transport of the oligomer in the skin viable tissues. Since the self-decrease appears only when PS is present during the electric treatment, PS must be present in the pathways during the pulses to prevent the post-pulsing transport. The pulses might modify the physicochemical properties of the inside of the pathways, what could potentialize the interaction (Vanbever et al., 1997b).

4.3. Targeting of PS in the skin viable tissues

We showed above that PS is transported in the skin viable tissues only during pulses. This creates a reservoir of PS in the target tissues, which persists within a therapeutic range (around 1 μM) for hours (Fig. 3). However, the transdermal passage of the oligomer is very low after the electric treatment (less than 3 pmol/cm^2 are found in the receptor compartment 4 h after the pulses (data not shown) vs 30 pmol/cm^2 in the skin viable tissues (Fig. 2)). This low tendency of PS to diffuse out of the skin after electroloading is favorable for topical applications, since it is desirable to keep the ON in the skin and to avoid diffusion into the systemic vasculature to minimize potential side effects (Shah et al., 1992).

4.4. Iontophoresis vs electroporation

The efficiency of the electrophoretic transport of ON to the skin viable tissues is equivalent for iontophoresis and electroporation (Fig. 4). In the stratum corneum, in contrast, the quantity of oligomer is more important after electroporation than after iontophoresis. As the routes of electrically facilitated flow are different according to the method of delivery used (mainly appendages for iontophoresis (Guy, 1992), versus intercellular and/or transcellular pathways for electroporation (Pliquett et al., 1996; Prausnitz et al., 1996b; Vanbever, 1997)), the higher quantity of PS found in the stratum corneum after electroporation could have two interpretations: (i) electroporation might allow PS to reach and bind more easily to some molecular species of the stratum corneum (e.g. intracellular keratin), and, as stated above, could moreover modify the pathways; and/or (ii) steric hindrance in the stratum corneum during pulsing might be stronger than during iontophoresis due to the pathways shape and/or size. The interaction of the molecule transported with the inside of the pathways would depend on the physicochemical properties of both the drug and the pathway. For both methods of delivery, the net quantities transported across the stratum corneum would depend on: (i) electrophoresis; (ii) steric or electrostatic hindrance in the pathways; and (iii) affinity for the pathways. The balance between those parameters should vary according to the molecule transported or the electric treatment, leading to different results.

Finally, given that iontophoresis, contrary to electroporation, is not believed to permeabilize lipid membranes (Prausnitz et al., 1996a), the localization of the oligomer within the skin tissues may differ according to the method of delivery used. Indeed, PS localization within the skin tissues might differ in terms of depth of permeation or skin area involved in transport, but also in terms of localization at the cellular level (Regnier et al., 1996). Only electroporation permeabilizes the skin cells, facilitating intracellular uptake of the antisense compound (Regnier and Préat, accepted).

5. Conclusions

We previously showed that the quantity of PS delivered to the skin viable tissues 4 h after pulsing can be controled by the electric parameters of the pulses, the total charge transferred or the concentration of PS in the donor solution. PS is not degraded in the skin. The levels of PS in the viable tissues are potentially therapeutic (Regnier et al., 1998). This paper shows that only a few minutes are needed to achieve such concentrations in the viable tissues, electrophoresis creating a long-lasting reservoir of PS in the skin viable tissues during the pulses. Prolonged passive diffusion after electroporation was thus not required for efficient topical delivery of PS.

The major mechanism of ON transport by electroporation was electrophoresis. Thus, for the optimization of ON delivery, PS should be delivered from the cathodal compartment with a low ionic strength donor solution.

This study also shows that PS has great affinity for the stratum corneum, and seems to interact strongly with the pathways during the electric treatment. Therefore, it might be interesting to envisage other oligonucleotide derivatives than phosphorothioate (e.g. 3′ end protected phosphodiesters), to improve the permeation of the oligomers in the skin.

Finally, localization studies have to be performed to pursue the comparison between iontophoresis and electroporation, which both deliver equivalent amounts of PS.

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