Mechanisms Involved in Iontophoretic Transport of Angiotensin

Martine Clemessy,[1] Guy Couarraze,[1,3] Bruno Bevan,[2] and Francis Puisieux[1].

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Purpose. The feasibility of using iontophoresis to enhance the permeation rate of a model peptide was investigated in vitro using hairless mouse skin. ***Methods.*** Angiotensin 2 (AT 2) was employed as a permeant probe, using optimum iontophoresis conditions. A number of physicochemical parameters (donor ionic strength; valence of competitive ions; pH of donor solution) were studied with the aim of exploring the mechanisms involved in the iontophoretic transport through the skin: electrokinetic transport or convective transport. For this purpose, the magnitude of the convective solvent flow was also evaluated by the permeation of (^{3}H) H_2O. The interest of pulsed currents for peptide delivery was also investigated and the effect of current density and frequency was studied. ***Results.*** AT 2 transport was found to be enhanced 20-fold in comparison to passive permeation and was found to be proportional to the current density with direct currents as with pulsed currents. ***Conclusions.*** Although the flux enhancement of ions during iontophoresis is due principally to the electrical potential gradient, secondary effects such as convective solvent flow contribute also to flux enhancement of peptide delivery. This effect is dependent of physicochemical conditions of formulation.

KEY WORDS: iontophoresis; iontohydrokinesis; angiotensin 2; transdermal delivery; direct current; pulsed current.

INTRODUCTION

Numerous literature reports have described enhanced delivery of a variety of peptides across biological membranes by means of iontophoresis (1–3). In this paper, following a brief evaluation of experimental conditions of iontophoretic delivery of a model peptide, the underlying mechanisms of peptide transport during iontophoresis are examined. In particular, although the theoretical Nernst-Plank description assumes that the iontophoretic flux is controlled entirely by the primary driving force (the electrochemical potential gradient), secondary effects (such as convective solvent flow induced by currents) may also contribute to iontophoretic flux enhancement. The term "iontohydrokinesis" (IHK) has been adopted to describe water transport during iontophoresis, but no specific mechanism has been attached to this new term (4).

Several mechanisms are generally evoked to explain the water convective movement responsible for IHK, such as electro-osmosis and transport number effect.

Electro-osmosis. The skin is a negatively-charged membrane when exposed to a solution with a pH greater than 4. Thus the pores of the skin contain a double layer of positively charged water. Under an electrical field, counter-ions present in this double layer will carry away water molecules by a mechanical effect.

Transport Number Effect. as the transport number of Na^+ and Cl^- in the skin are different from their free solution values, a current-induced concentration gradient of these physiological ions is created across the skin, inducing an osmotic flow of water.

In the present study the contribution of iontohydrokinesis to the flux enhancement of a small peptide during iontophoresis has been examined. For this purpose angiotensin 2 was used as a permeant probe.

MATERIALS AND METHODS

Diffusion Cell

The static permeation cells used in this study were made up of a donor compartment and a receptor compartment separated by a skin sample. The donor compartment was filled with the peptide solution or tritiated water and contained an Ag/AgCl disc electrode (1.77 cm^2) connected to the positive pole of a current generator. The receptor compartment (12 ml) was filled with a 0.9% NaCl isotonic solution and contained an Ag/AgCl wire electrode connected to the negative pole of the generator. A mixture of antibiotics (penicillin-streptomycin solution; Sigma) was added in the receptor compartment in order to maintain the hairless mouse skin for 48 hours without degradation. The permeation cells were maintained in a thermostated water-bath at 37°C. For each experiment, 6 permeation cells were used.

Donor Solutions

Angiotensin 2 (Sigma) (Asp^-—Arg^+—Val—Tyr—Ile—His^+—Pro—Phe at pH 6.5), molecular mass 1046, pH_i = 7.7

^{3}H Angiotensin 2 solution	
Angiotensin 2	0.01%
^{3}H Angiotensin 2 (Amersham)	6.25 μCi/g
NaCl	0.7%

(This concentration was determined in order to allow an oxidation reaction at the Ag/AgCl anode during 5 hours of iontophoresis without bringing an excess of competitive ions).

$^3H\ H_2O$ solution

^{3}H Angiotensin 2 was replaced by tritiated water (Dositek, France) in order to achieve an activity of approximately 4 μCi/g.

Skin. Abdominal hairless mouse (Iffa Credo- France) skin was used.

Assays. Samples containing labelled substances and scintillation liquid were counted in a β spectophotometer scintillation counter (Beckman LS 6000)

Current Generator. The current generator can provide

[1] Centre Pharmaceutique, URA CNRS 1218, Université Paris-Sud, 92290 Châtenay-Malabry (France).
[2] Laboratories Fournier SC.A., division Tilderm systems, 21300 Chenove (France).
[3] To whom correspondence should be addressed.

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pulsed or direct currents stabilized to the same intensity value to each of 6 permeation cells.

RESULTS AND DISCUSSION

Iontophoresis Characteristics

Permeation enhancement of Angiotensin by direct current (0.28 mA/cm²)

Angiotensin 2 (AT 2) was chosen to illustrate the effects of iontophoresis because it may be regarded as a stable model molecule for peptide delivery. Preliminary studies were made with Angiotensin 1 (molecular mass 1296) and Angiotensin 2 and the radioactive derivates ^{125}I AT1 (N.E.N.) and ^{3}H AT2 necessary for an accurate detection in the receptor compartment of diffusion cells. After 10 hours of direct current (0.5 mA) in a solution of unlabelled peptide (0.01%) chromatograms obtained by H.P.L.C. did not show any degradation peak, for either peptide. The radiochemical stability of labelled molecules was evaluated under the same conditions by thin layer chromatography. A migration spot, attributed to iodide ions by comparison with a control of sodium iodide, was observed with ^{125}I AT1, whereas no supplementary peak was obtained with ^{3}H AT2. Thus the chemical and radiochemical stability of AT2 has been confirmed in our experimental conditions, and this molecule was retained for the study.

The electrical current density (0.28 mA/cm²) was chosen because it is generally recognized that skin tolerates a current density lower than 0.5 mA/cm². The cumulative amount of solute permeating is plotted against time for a variety of conditions. Figure 1 shows a typical graph for the iontophoretic and passive diffusion of AT 2 under standard experimental conditions. The delivery of angiotensin 2 by iontophoresis with Ag/AgCl electrodes required the addition of chloride ions in the donor medium. Despite the fact that AT 2 is not the major charge carrier in the solution (NaCl concentration 0.7%, AT 2 concentration 0.01%), a 20-fold increase of delivery was obtained since flux increased from 0.011 ± 0.006 nM/cm².h (passive permeation) to 0.226 ± 0.036 nM/cm².h.

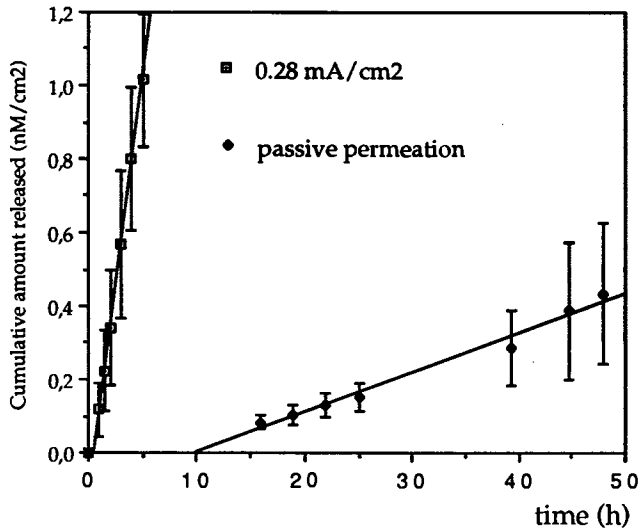

Fig. 1. Kinetics of Angiotensin (AT 2) permeation during iontophoresis at 0.28 mA/cm² and without electrical current (paasive permeation) (n = 6; error bar = 1 SD).

A comparison of the lag times between the passive permeation kinetics (10 hours) and the iontophoretic permeation kinetics (0.5 hours) yields the same factor: 20. The iontophoretic transport of a peptide involves a great number of parameters. If we consider the electrokinetic effect alone, the valence of the peptide, the competition between peptide and other ions present in the donor compartment, the ionic strength and the valence of the competitive ions as well as electrical parameters must be considered. However, if the drug ion is present in very small amounts in comparison with other ions having a higher mobility, iontohydrokinesis could be involved in the transport of the drug ion through the skin.

Evidence of Electro-Osmosis

Since electro-osmosis is often considered as an important mechanism of peptide iontophoretic delivery, we measured the permeability of skin to tritiated water during a passive experiment and during an iontophoretic experiment (0.28 mA/cm²). The results show a 4-fold increase of water flow, this corresponds to the demonstration of the existence of a solvent convective flow responsible for iontohydrokinesis effects. The values of the water flow (2.16 ± 0,5 µl/cm². h for passive permeability and 8.41 ± 3.1 with 0.28 m A/cm² direct current) are of the same magnitude as the water flux evaluated by several other authors (4–6).

The demonstration of water movement under an electrical field reveals the iontohydrokinesis phenomenon in our experimental conditions, but does not allow us to draw conclusions about the magnitude of the contribution of IHK to the transcutaneous passage of AT 2.

The same current induces an enhancement factor of 20-fold on AT 2 flux. These two results are in favour of the predominance of an electrokinetic mechanism for the transport of peptide even if the existence of electro-osmosis has been demonstrated.

Effect of Intensity

In the literature, the relationship between current intensity and drug flux has been demonstrated for several species: morphine (7); salbutamol (8); lidocaine (9), but usually the drug under investigation is the major ion in the solution. In our case, the concentration of AT 2 is very low compared to the concentration of competitive ions; moreover, electro-osmosis is probably a significant mechanism involved in the transport of the peptide. Thus, we have to verify whether the modulation of the fluxes as a function of the current occurs in our model. In order to eliminate variability in skin permeability, we have applied 3 different current densities to the same skin sample. (0.08; 0.17 and 0.28 mA/cm²), and the duration of each step was reduced to 2 hours in order to avoid any risk of skin alteration.

Figure 2 shows the relation between the electrical current and AT 2 fluxes. An exact modulation of AT 2 fluxes is obtained, since the iontophoretic efficiency (2.310^{-3}; 3.10^{-3}; $2.9\ 10^{-3}$ respectively) is fairly constant for the three intensities, although 99.7% of the charges are transported by ions other than AT 2. When the current density rose from

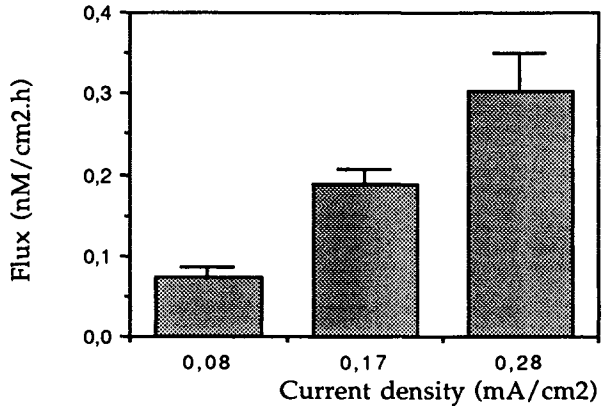

Fig. 2. Angiotensin fluxes at different current density: 0.08; 0.17 and 0.28 mA/cm^2 (n = 6; error bar = 1 SD).

0.08 to 0.28 mA/cm^2, the water flow only increased from 4.37 ± 0,5 to 6.95 ± 0.9 µl/cm^2/h. This is further evidence that peptide transport is not only convective because the increase of the current density from 0.08 to 0.28 mA/cm^2 leads to a 1.6-fold increase of electro-osmosis flow, while AT 2 flux increases 4-fold.

Donor Composition

Donor Ionic Strength

If the iontophoretic delivery of AT 2 is not due to a pure electrokinetic displacement alone, modification of one experimental parameter will lead to an effect on AT 2 flux in proportion to the mechanisms involved in electrokinetic transport and iontohydrokinesis. In addition, the evaluation of the water flow through the skin should give more precise information about mechanisms involved. Thus water flow was systemically evaluated under the same experimental conditions as AT 2 flux evaluations.

The NaCl content of the donor solution was modulated without changing the amount of AT 2 and 3 different concentrations (0.06; 0.12 and 0.24 M/L) were considered. Figure 3 shows a regular decrease of AT 2 flux with the increase in ionic strength. This result can be interpreted by a simple increase of the electrokinetic competitive effect between positively charged peptide and Na^+ ions.

Figure 4 shows the water fluxes under the same conditions: the highest flux is observed at 0.12 M/L, while the highest AT 2 flux is at 0.06 M/L. It is noteworthy that AT 2 flux does not vary as a function of ionic strength in the same way as the water flux, *this is not in favour of transport by a pure electroosmotic effect.*

The evolution of the water flow with the ionic strength could be explained if we consider that water flow arises from many mechanisms which may or may not require an electrical current: osmosis and electro-osmosis. At 0.06 M/L, the donor medium is hypotonic, so an osmotic flow from donor to receptor is created. When the ionic strength of the donor is increased, the osmotic flow will decrease. At 0.24 M/L, the donor is hypertonic, the water flow will then be from receptor to the donor. On the contrary, as the ionic strength is increased, the part of the electrical current transported by Na^+ increases, resulting in an increase in the convective water flow from donor to the receptor.

This analysis shows how the interpretation of the evolution of water flow with the ionic strength is complex and involves mechanisms acting in opposite directions. Thus, the maximum level of water flow for a NaCl concentration of 0.12 M/L could be the result of several mechanisms acting in opposite directions.

Influence of Valence of the Competitive Cations on AT 2 Flux

Since the competition between the drug and cations present in the donor medium is an important factor in iontophoretic transport, the addition of chloride ions to the donor medium by $CaCl_2$ instead of NaCl can provide interesting information. Ca^{++} has a smaller transport number than Na^+. Thus competition between the drug and Ca^{++} might be lower and angiotensin delivery could be increased. AT 2 flux was significantly higher ($p = 0.01$; Student test) in presence of NaCl than with $CaCl_2$, whatever the pH value (cf Table I), although the former salt should have induced a larger competitive effect.

Water flow measured under the same conditions shows the same effect: water flux in presence of NaCl is 1.7-fold higher than in presence of $CaCl_2$. Thus, in this case a con-

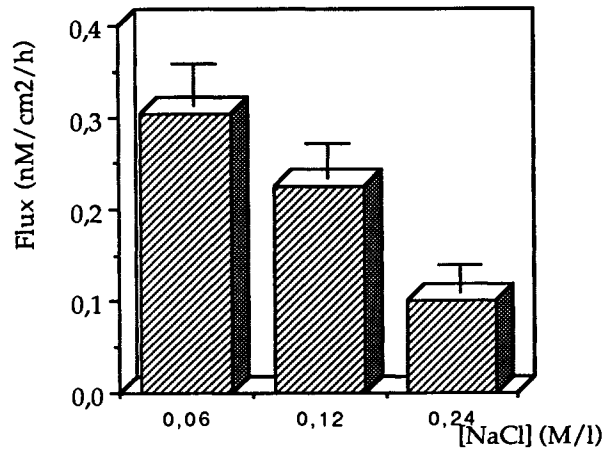

Fig. 3. Angiotensin flux at 0.28 mA/cm^2 versus donor ionic strength (n = 6; error bar = 1 SD).

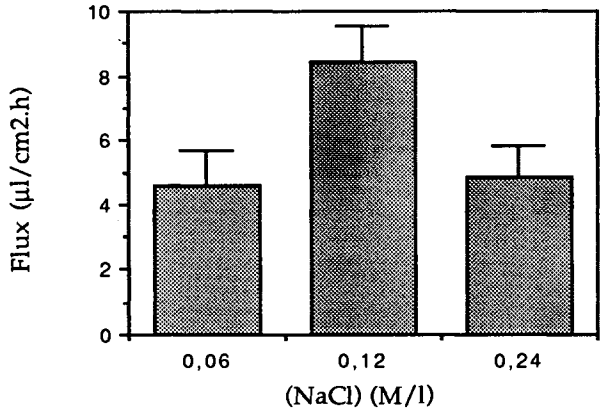

Fig. 4. Water flow at 0.28 mA/cm^2 versus donor ionic strength (n = 6; error bar = 1 SD).

cordance between AT 2 fluxes and water fluxes was observed.

On the contrary, our result could suggest a participation of convective transport: thus, if multivalent cations are able to neutralize a part of the negative charge of the skin, the cation permselectivity of the skin and the size of the double layer will decrease and as a result the water flow due to iontohydrokinesis will also decrease. This result is in agreement with the result of Burnette and Ongpipatanakul (10): they observed that the mannitol flux was lower when Ca^{++} is present in the donor compartment than when Na^{+} is present.

Influence of donor pH on fluxes

The pH of the donor medium could give rise to 2 different effects:

- Since peptide ionization is pH-dependent, the pH value sets the charge level of the peptide and acts directly on the electrokinetic transport.
- As the electrical charge of the skin is pH dependent, the pH value acts indirectly on the mechanisms involved in the transport of ions and water in the skin.

Three NaCl solutions of different pH but of same ionic strength were investigated: pH = 4.5; 6.5 and 8.5. Two $CaCl_2$ solutions of different pH but of same ionic strength were also investigated: pH = 4.5 and 6.5. Since the isoelectrical point of AT 2 is 7.7, the peptide carries one positive charge at pH values below 7.7, and AT 2 has no residual charge at pH 8.5.

Table I shows AT 2 flux variation with pH for NaCl solutions. At pH 4.5, AT 2 flux (0.171 nM/cm^2.h) is smaller than at pH 6.6 and 8.5 (respectively 0.226 and 0.219 nM/cm^2.h). This is surprising since the ionized fraction of AT 2 is higher at pH 4.5. Table I shows that this result was confirmed with $CaCl_2$, and the same ratio (0.76) between pH 4.5 flux and pH 6.5 flux was obtained.

Similar results have been observed previously with human skin and hairless mouse skin. Burnette and Marrero (1) showed that iontophoresis of thyrotropin releasing hormone was greater at pH 8 than at pH 4 although the peptide had a net positive charge at pH 4 but was effectively neutral at pH 8. Robert et al. (11) showed with human skin that iontophoretic absorption of Amphotericin (pK_a 5.5 and 10) as a singly-charged cation (pH 3.5) was only slightly faster than as a zwitterion (at pH 7.5). In contrast, Green et al. (12)(13) found that histidine and a tripeptide were more efficiently iontophorized at pH 4 (net charge = +1) than at pH 7.4 (nonionized). The interpretation of all these results is complicated by inconsistencies in the experimental conditions employed by different authors. There are interacting factors like differences in electrolytes (transport number; ionic strength . . .) and in pH values.

Table I. Angiotensin Flux (mean ± SD, n = 6) at 0.28 mA/cm^2 Versus pH with Donor Medium Containing NaCl or $CaCl_2$

	pH	AT 2 flux nM/cm^2.h
NaCl	4.5	0.171 ± 0.07
NaCl	6.5	0.226 ± 0.04
NaCl	8.5	0.219 ± 0.05
$CaCl_2$	4.5	0.067 ± 0.02
$CaCl_2$	6.5	0.090 ± 0.05

Consequently, if the major effect of the pH is not correlated to the electrical charge of the peptide, then the pH could act on the iontohydrokinesis flow. This is confirmed by the tritiated water flow under the same experimental conditions. A smaller water flux was observed at pH 4.5. The ratio (0.63) between the water flow at pH 4.5 and 6.5 has the same magnitude as the peptide flux ratio.

The fact that at pH 4.5 the water flow is below the flow at pH 6.5 is consistent with a decrease in the cation permselectivity of the skin due to a progressive neutralization of a part of the negative charges of the skin, and with a decrease in the double layer of counterions, both resulting in a decrease of the convective flow from anode to cathode.

This is consistent with the results of Kim et al (14): when the pH of the donor and receptor solutions was buffered at slightly less than 4, Kim et al. showed that there was a net volume flow from cathode to anode, indicating that the permselectivity of the membrane had been changed. However, they also showed that when a pH gradient was maintained across the skin (donor pH near 4, receptor pH 7.4), the electrotransport behavior was more complex but, ultimately, resulted in retention of the skin's inherent cation permselectivity.

The correlation between AT 2 fluxes and ^3H_2O flux versus pH shows that iontohydrokinesis could be responsible for a significative part of the transport of angiotensin. The previous results showing that under other conditions angiotensin flux and water flux are not correlated, lead us to the following conclusion: even if the contribution of convective transport has been demonstrated, this mechanism is not the only mechanism involved in the percutaneous transport of angiotensin by iontophoresis.

Pulsed Currents

Pulsed currents has been reported to have a great potential in iontophoresis in order to better preserve skin integrity. It has been demonstrated that pulsed currents do lead to a better stability of skin permeability than a direct current of same current intensity. In a previous report (7) it was shown that the application of a direct current of 0.28 mA/cm^2 during 5 hours led to a significative increase in tritiated water flow, whereas a pulsed rectangular current (on/off ratio: 1/1; frequency 1000 Hz; current density 0.28 mA/cm^2) allowed the return to the initial passive permeability. Thus, it was important to verify whether a pulsed current could lead to the delivery of AT 2 and whether fluxes could be modulated by the current density under these conditions.

Table II shows a good correlation between AT 2 fluxes and current density for a rectangular current with 1 kHz frequency and on/off ratio 1/1. On the other hand, the iontophoretic efficiencies for AT 2 delivery are comparable to those obtained with direct currents of the same intensity. Thus, the iontophoretic permeability of AT 2 is not increased by direct current in comparison to pulsed current, although tritiated water permeability is increased. This result could be interpreted by the difference in size (steric hindrance) be-

Table II. Angiotensin (AT 2) Fluxes (mean ± SD, n = 6) and Iontophoretic Efficiency at Different Current Densities Using Pulsed Rectangular Currents (on/off Ratio: 1/1; Frequency: 1000 Hz)

Current density	Flux (nM/cm^2.h)	Efficiency (10^{-3}.%)
0.08 mA/cm^2	0.042 ± 0.016	2.66
0.17 mA/cm^2	0.066 ± 0.014	2.09
0.28 mA/cm^2	0.100 ± 0.019	1.90

tween the two molecules: the modification in cutaneous permeability induced by direct current would mainly concern pathways used by the smallest molecules. This shows the interest of using pulsed current for peptide delivery: in fact pulsed current allows the skin integrity to be respected without any decrease of the peptide permeability.

Frequency is, like intensity, an important parameter of pulsed currents. Table III shows AT 2 fluxes as a function of frequency from 0.5 to 15 KHz. AT 2 fluxes increase 3.5-fold when the frequency is increased from 0.5 to 15 KHz. This result could be useful for the conception of an iontophoretic device and could be explained by a decrease in skin impedance with the increase in current frequency as shown by the skin equivalent circuit model of Yamamoto and Yamamoto (16) for example. This result is in agreement with the results obtained in vivo with other peptides such as insulin (17).

CONCLUSIONS

Angiotensin, considered as a small model peptide, can be delivered percutaneously by iontophoresis. Fluxes of angiotensin are very well modulated by the current intensity.

The effect of formulation parameters such as pH, ionic strength or the nature of competitive ions, are complex because of the involvement of several mechanisms of transport allowing the displacement of the drug by an electrokinetic force or an electroconvective flux of water. The contribution of electro-osmosis has been proved by measurement of tritiated water flux. The interest of pulsed current leading to a better respect of skin integrity has been demonstrated since the iontophoretic efficiency of angiotensin transport through the skin was similar to that obtained with direct current.

Table III. Angiotensin (AT 2) Fluxes (Mean ± SD, n = 6) and Iontophoretic Efficiency at Different Frequency (Pulsed Rectangular Current: 0.28 mA/cm^2—on-off ratio 1/1)

Frequency	Flux (nM/cm^2.h)	Efficiency (10^{-3}.%)
0.5 KHz	0.041 ± 0.017	0.78
1 KHz	0.043 ± 0.011	0.81
5 KHz	0.062 ± 0.024	1.18
15 KHz	0.091 ± 0.024	1.73

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