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The viability of isolated rabbit nasal mucosa in the Ussing chamber, and the permeability of insulin across the membrane

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Summary

The viability of isolated rabbit nasal mucosa (lateral wall) and the permeability of insulin across the membrane have been tested in an Ussing chamber. The short-circuit current (I_{sc}) was relatively stable between 1 and 10 h. Average I_{sc} for this period ($I_{sc,1-10}$) was 92 ± 13 $\mu A/cm^2$. The viability of the tissue was longer than 10–12 h, where I_{sc} was about 85 and 66% of $I_{sc,1-10}$, respectively. During 1–10 h the average potential difference (PD) was 6.4 ± 1.5 mV (mucosal side was negative) and the average tissue resistance (TR) was 69 ± 8 Ω cm^2. The appearance of insulin at the serosal side is expressed as ‰ of the initial insulin concentration (8 mg/ml) at the mucosal side. Mean appearance rate was 0.44 ± 0.34‰ h^{-1}, equivalent to an apparent penetration coefficient (P_{app}) of 0.24×10^{-6} cm/s. The same value, corrected for possible degradation and/or adsorption at the serosal side, is 0.73 ± 0.42‰ h^{-1} ($P_{app} = 0.4 \times 10^{-6}$ cm/s). The concentration of insulin at the mucosal side between 5 and 90 min was relatively constant, the average recovery being about 85%. The method is found to be useful for physiological and toxicological studies. Whether it is useful to screen absorption enhancers for peptides remains to be confirmed.

Introduction

At present there is a great and increasing interest for systemic application of drugs via the nasal cavity. Some of the reasons are that the first-pass effect is avoided, the time to effect may be close to i.v. and it is painless and easy to administer. The potential for intranasal application of peptides is especially large, as the leaky ordered pseudostratified epithelium is relatively permeable to large molecules, and local enzymatic degradation is less likely to take place than, e.g., in the gastrointestinal tract. For most peptides, however, there is normally a need for coadministration of a penetration enhancer and/or an enzyme inhibitor to increase the absorption.

Absorption of drugs from the nasal mucosa can be examined in many ways (Gizurarson, 1990). Formulations have, e.g., been tested in vivo in humans (Hussain et al., 1980a) as well as in rabbits (Sørensen et al., 1988), rats and dogs

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(Hussain et al., 1980b). In vitro experiments have used isolated nasal mucosal tissue from rabbits (Melon, 1968; Gizurarson et al., 1991) and humans (Cotton et al., 1987; Gizurarson et al., 1991) mounted in an Ussing chamber for electrophysiological measurements. Absorption of drugs and enhancer toxicity have, e.g., been studied in isolated nasal mucosa from sheep (Wheatley et al., 1988).

The number of peptide penetration studies using the Ussing chamber system are still limited. This type of studies have for example been performed with isolated nasal mucosa from rabbits (Hersey and Jackson, 1987; Cremaschi et al., 1990; Uchida et al., 1991) and rectum (Lee et al., 1988). In pilot studies using isolated human vaginal tissue and vasoactive intestinal peptide Bechgaard et al., (1992) introduced a method to correct and prevent enzymatic degradation of peptides and/or adsorption to the surroundings.

The purpose of the present paper is to study the viability of the isolated rabbit nasal mucosa in the Ussing chamber system, to describe a method to correct for enzymatic degradation and/or adsorption to surfaces and to investigate the permeability of insulin through the membrane.

Materials and Methods

Chemicals

Zinc-free human insulin was kindly provided by Novo Nordisk A/S (Bagsværd, Denmark). The bicarbonate Ringer solution (GR) consisted of HPO_4^{2-} (1.6 mM), $H_2PO_4^-$ (0.4 mM), Mg^{2+} (1.2 mM), Cl^- (122 mM), Ca^{2+} (1.2 mM), K^+ (5 mM), HCO_3^- (25 mM) and Na^+ (141 mM); all chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) and D-(+)-glucose (13 mM) was supplied by May & Baker (Dagenham, U.K.). Bovine serum albumin (BSA), RIA grade, was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dissolved in GR (1 and 3 mg/ml). 5 μCi/ml ^{14}C-labelled polyethylene glycol 4000 (PEG 4000) is commercially available from New England Nuclear (Du Pont, Boston, MA, U.S.A.). Phosphoric acid and anhydrous sodium sulfate were of analytical grade and obtained from Merck (Darmstadt, Germany). Acetonitrile of HPLC grade was purchased from Rathburn (Walkerburn, U.K.). Carbogen (95% O_2/5% CO_2) was obtained from Dansk Ilt & Brint fabrik A/S (Ballerup, Denmark). Scintillation cocktail (Pico-aqua) was obtained from

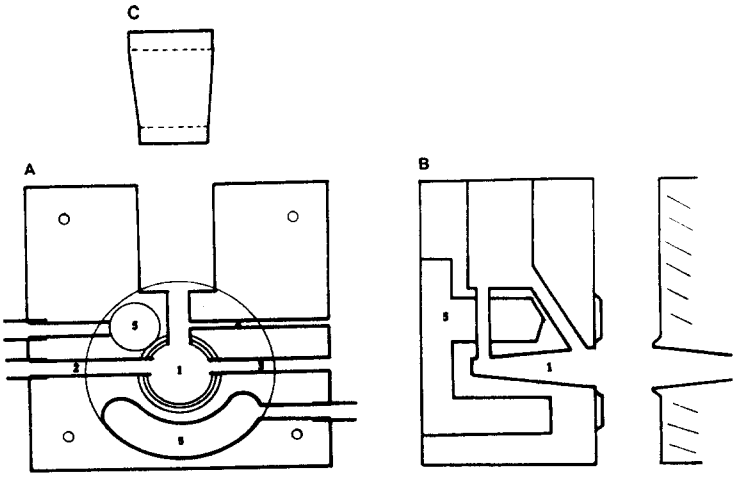

Fig. 1. Cross-section (A), side view (B) and funnel (C) of the Ussing chamber. (1) Chamber (1 ml). (2) Glucose-Ringer agar bridges which connect the voltage sensing electrodes to the bathing compartments. (3) Current-sensing electrodes (Ag/AgCl). (4) Gas lift system to provide oxygenation and circulation (95% O_2/5% CO_2). (5) Water jacket (37°C). The aim of the funnel is to prevent overflow.

Packard Instrument BV (Groningen, The Netherlands).

Apparatus

The Ussing chamber (Fig. 1) consisted of two 1 ml acrylic half-chambers obtained from the Zoophysiological Institute (August Krogh Institute, University of Copenhagen, Copenhagen, Denmark), connected to calomel electrodes (K401 Calomel electrode) from Radiometer A/S (Copenhagen, Denmark), a DVC-1000 dual voltage clamp (World Precision Instruments Inc., CT, U.S.A.) and Lauda MS temperature adjustable water bath (Messgeräte-Werk Lauda, Lauda-Königshofen, Germany). The electrical characteristics were registered by a BBC SE120 recorder (Goerz Electro Gmbh, Vienna, Austria).

The Hitachi HPLC system, used for quantitative analysis of insulin, consisted of a 655A-1 pump, a 655A variable-wavelength UV detector and a Rheodyne® (Berkeley, CA, U.S.A.) 7125 injection valve, equipped with a 20 μl loop. The column was a 4 × 250 mm LiChrosorb® RP-18 (5 μm) and the guard column was a LiChroCART® 4-4 from Merck (Darmstadt, Germany).

The scintillation counter was a PL Tri-Carb from Packard Instruments Co. (Downers Grove, IL, U.S.A.).

Analysis

Insulin was analyzed by a HPLC method modified from the procedure described by Snel et al., (1987). The mobile phase was a mixture of two solutions A and B (10 + 9). Solution A (pH 2.5) consisted of 0.04 M phosphoric acid, 0.2 M sodium sulfate and 10% acetonitrile. Solution B was a 50% acetonitrile/water solution. Detection was performed at 214 nm. Flow rate was 1 ml/min, column temperature 25°C and injection volume 20 μl. The retention time (t_r) was about 7 min. Sample concentration was calculated on the basis of peak height × t_r relative to external insulin standards (8000 μg/ml in GR and 40 μg/ml in 0.3% w/v albumin-GR solution (GRA)).

The detection limit was about 0.25 μg/ml and the precision about 5%.

Samples from the mucosal side were diluted 1 + 80 with mobile phase.

Tissue preparation

Male and female New Zealand White rabbits, weighing 2.5–3.0 kg were obtained from Novo Nordisk's own stock. They were killed by injection of about 10 ml 5% pentobarbitone into the marginal ear vein.

Immediately after death, a longitudinal incision through the lateral wall was made and the nasal cavity was fully opened. The middle and ventral nasal conchae were removed. The tissue was isolated by cutting along the whole septum then carefully loosening the mucosal tissue from the underlying cartilage and bone. The tissue was immediately placed in oxygenated GR.

Ussing chamber set up

The freshly isolated tissue was mounted between two acrylic half-cells (0.50 cm^2 exposed surface area) which were joined to form the complete Ussing chamber (Fig. 1). The reservoirs were equipped with a gas lift system (carbogen) to provide both oxygenation and circulation of the chamber solution. The total volume of each reservoir was 1.0 ml and the whole system was maintained at 37°C throughout the study.

Viability study

The electrophysiological properties of the rabbit nasal tissue were registered to establish the tissue viability over a period of 12 h. 1 ml GR and 1 ml 0.1% GRA were added to the mucosal (M-side) and serosal (S-side) side, respectively. To register whether the decline in potential difference (PD) and short-circuit current (I_{sc}) after 12 h were due to lack of glucose in the media, 400 μl of the fluid in each reservoir were replaced with fresh 37°C GR and 0.1% GRA, respectively.

Penetration study

The tissue was preincubated 60 min for stabilisation. GR and 0.3% GRA were added to the M- and S-side, respectively. During the preincubation period and the penetration study the PD and

I_{sc} were measured. After preincubation, 500 μl GR were replaced with an equal volume of GR solution containing 16 mg insulin/ml.

Samples (25 μl) for insulin analysis were withdrawn from the M-side after 5, 25, 45 and 90 min and diluted (1 + 80) with HPLC mobile phase, and from the S-side after 0, 15, 35, 55, 70 and 85 min. The sample volumes were replaced with GR and 0.3% GRA, respectively.

Disappearance study

To determine the possible disappearance rate of insulin from the S-side 40 μg insulin were added to the S-side immediately after the 90 min penetration study. 25 μl samples were withdrawn after 6, 16 and 31 min for analysis.

Tissue control

Each experiment was terminated by tissue control. First, a blank sample (100 μl) from the serosal fluid was removed then the serosal half-chamber was refilled to 1 ml with 0.3% GRA solution. The tissue integrity was tested by replacing 30 μl GR with 30 μl PEG-4000 at the mucosal side of the Ussing chamber. 15 min later 100 μl samples were collected from both half-chambers. The samples were mixed with 4 ml scintillation cocktail and the radioactivity was counted for 5 min.

Calculation

The concentration of insulin during the transport study was corrected for dilution using the following equation:

$$Q = V_s\left(\sum_{n=1}^{n} C_{n-1}\right) + C_n V_t$$

where Q is the total amount of insulin, V_s denotes the sample volume, V_t is the chamber volume and $C_{1,2,\ldots,n}$ represents the concentration of sample $1,2,\ldots,n$.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{X_2}{X_1}\frac{V_2}{AT}$$

where X_2 and V_2 are concentration and volume at the serosal side, respectively, and X_1 denotes the concentration at the mucosal side. A represents the surface area of the exposed nasal mucosal tissue and T is the time.

The appearance rate is equal to $\alpha \times 60$ min/h, where α is the slope of the regression line (see below).

The theoretical values are calculated by using linear regression at the measured values from 35

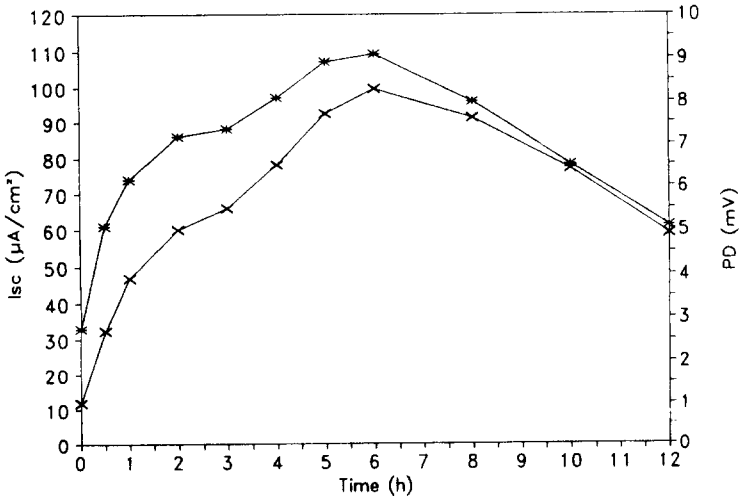

Fig. 2. Mean short-circuit current (I_{sc} (★——★)) and potential difference (PD (× —— ×)) at different times (n = 9).

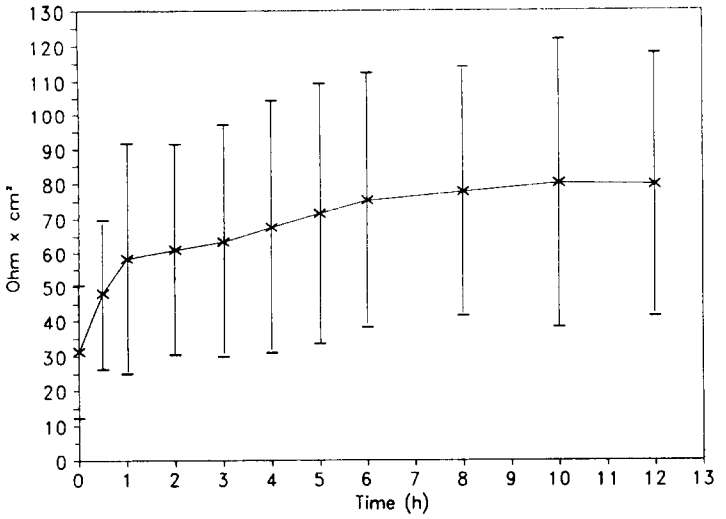

Fig. 3. Mean (± S.D.) transepithelial resistance (TR) at different times ($n = 9$).

to 85 min with the exception of Expt III where the range 55–85 min is used. The theoretically obtained value (after 90 min penetration study) is then equal to $\alpha \times t + 5‰$.

The correction for degradation and/or adsorption is calculated from the following equation:

$$\text{Corr}(\%/\text{h}) = (B - C)\frac{100}{1/2(A+B)} \times \frac{60}{t_b - t_a}$$

where A and B are the theoretical values at 90 and 121 min, respectively. C is the measured value at 121 min. t_a and t_b are the times corresponding to A and B, respectively.

Results

Fig. 2 shows that I_{sc} is relatively constant between 1 and 10 h. The mean I_{sc} for this period ($I_{sc,1-10}$) is 92 ± 13 $\mu A/cm^2$. The viability of the tissue is longer than 10–12 h, where I_{sc} is 85 ± 24

TABLE 1

Appearance of insulin at the serosal side in ‰ of the initial mucosal side concentration (8 mg / ml) at different times (min) during the experiment: at 90 min 5‰ insulin is added to the serosal side to estimate the correction for degradation

Expt no.	Serosal side (recovery ‰ at min)								Appearance rate	
	15	35	55	70	85	96	106	121	‰/h	(Corr.) [b]
I	0.01	0.01	0.00	0.00	0.00	4.14	3.88	3.18	0.00	–
II	0.07	0.23	0.37	0.46	0.57	5.36	4.91	5.19	0.40	0.48
III	0.03	0.05	0.06	0.16	0.24	4.76	4.96	4.47	0.36	0.52
IV	0.03	0.16	0.32	0.47	0.55	[a]	5.60	5.57	0.48	0.54
V	0.09	0.22	0.51	[a]	1.02	6.66	5.30	5.51	0.96	1.36
Mean	0.05	0.13	0.25	0.27	0.48	5.23	4.93	4.78	0.44	0.73
S.D.	0.03	0.10	0.22	0.23	0.38	1.08	0.65	1.00	0.34	0.42

[a] Not measured.

[b] Including the correction for degradation and/or adsorption.

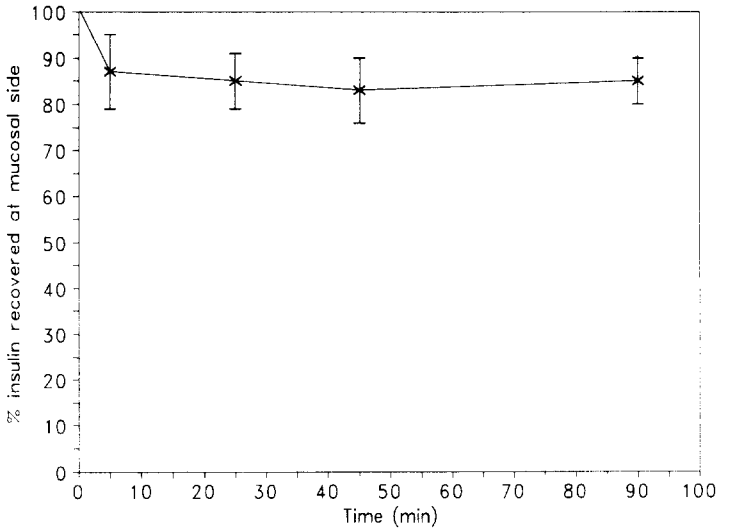

Fig. 4. Recovery (mean ± S.D., $n = 5$) of insulin at the mucosal side. The initial concentration is calculated from known amounts added (8 mg insulin/ml).

and 66 ± 22% of the $I_{sc,1-10}$, respectively. The maximum I_{sc} is observed after 6 ± 1 h, where I_{sc} is 110 ± 17 $\mu A/cm^2$. About 60 min after the nasal mucosa was mounted in the chamber, the membrane was relatively stable and ready to use.

Between 1 and 10 h the mean PD is 6.4 ± 1.5 mV (M-side negative) and the mean tissue resistance (TR) is 69 ± 8 Ω cm^2. The change in PD with time is depicted in Fig. 2, and the mean TR ± S.D. is shown in Fig. 3.

Table 1 shows the appearance of insulin (‰) at the S-side relative to the initial concentration at the M-side. Mean appearance rate is 0.44 ± 0.34‰ h^{-1}. The same value, corrected for possible degradation and/or adsorption (corr.) at the S-side, is 0.73 ± 0.42‰ h^{-1}.

Fig. 4 illustrates that the concentration of insulin at the M-side is relatively constant between 5 and 90 min, with mean recovery about 85%.

Discussion

The observed electrophysiological data ($I_{sc} \approx$ 90 $\mu A/cm^2$, PD ≈ 6 mV and TR ≈ 70 Ω cm^2) are very similar to the in vitro electrophysiological properties of isolated human nasal turbinate mucosa (about 60 $\mu A/cm^2$, 6 mV and 100 Ω cm^2; Cotton et al., 1987) and sheep nasal mucosa (about 150 $\mu A/cm^2$, 15 mV and 100 Ω cm^2; Wheatley et al., 1988). The observed PD (about 6 mV) is also very similar to the in vitro observations in rabbits (Melon, 1968; Gizurarson et al., 1991; Maitani et al., 1991). Unfortunately, I_{sc} and TR are not given in these papers. In vivo PD in normal humans has been registered to about 25 mV (Knowles et al., 1981; Gowen et al., 1988).

I_{sc} and TR may be considered more important than PD, as PD is only a function of the other two parameters. I_{sc} depends on the active sodium transport, and may therefore be used as an indicator for viability. A reasonable definition of viability may be the time where I_{sc} is more than 80% of the $I_{sc,1-10}$ value. In some cases a limit of, e.g., 50% may be acceptable. Based on this definition the viability is longer than 10–12 h, where I_{sc} is 85 and 66% of the $I_{sc,1-10}$ value, respectively. The viability is probably not limited from consumption of the glucose, as addition of solute glucose after 12 h did not change the I_{sc}. Wheatley et al. (1988) found, by measuring the electrical properties of the tissue, that sheep nasal mucosa

maintained viability up to 8 h even after response to ion transport modifiers.

TR remains relatively constant during the whole incubation period, which indicates that the tissue maintains its integrity. The observed TR (about 70 Ω cm^2) is remarkably low, equivalent to a relatively thin and/or leaky tissue membrane. Artursson (1990) observed a TR of about 250 Ω cm^2 in monolayers of cultured human intestinal cells (Caco-2 cells).

The observed penetration of 0.44‰ insulin/h is equivalent to an apparent penetration coefficient (P_{app}) of 0.24×10^{-6} cm/s. In isolated rabbit rectum, Lee et al., (1988) determined a P_{app} of 0.69×10^{-6} cm/s for insulin in combination with 1% sodium glycocholate as an absorption enhancer. Without enhancer no penetration was observed. In accordance with our observations, Hersey and Jackson (1987) found a P_{app} of 4×10^{-6} cm/s for the small cholecystokinin octapeptide. Relative to these results for peptides and tissue, Artursson (1990) reported an amazingly low P_{app} (0.2×10^{-6} cm/s) for the similar hydrophilic but small drug, atenolol, in monolayers of cultured human intestinal cells. This is consistent with the observed higher TR in cultured cells, but not directly expected for a monolayer. In vivo the absorption barrier practically only consists of a single layer of epithelial cells. In isolated mucosal tissue the barrier also consists of many underlying cell layers which should decrease the penetration, increase the TR and cause a penetration lag time. In our experiment we observed a lag time of about 15 min.

As an attempt to correct for possible enzymatic degradation and/or adsorption at the S-side, 40 μg insulin/ml (equivalent to 0.5% of the initial mucosal concentration) was added immediately after the 90 min transport study. The corrected appearance rate was 0.73‰, equivalent to a P_{app} of 0.4×10^{-6} cm/s, which is 67% higher than the uncorrected value. A correction in this case is therefore not important, indicating that the experiment is relatively well controlled with respect to degradation and adsorption, even at low concentrations. This is in agreement with the observations by Gizurarson and Bechgaard (1991) that intranasal degradation of insulin is low. Furthermore, no adsorption of insulin to the chamber walls from the 0.3% GRA solution was observed.

It is not clear whether the adsorption to the tissue is part of the correction. At least it appears to explain the initial fall of about 15% in the insulin concentration at the M-side (Fig. 4).

Albumin is added to prevent adsorption from low peptide concentrations. It is only added to the S-side, since addition to the M-side may interfere with the absorption rate. 0.3% albumin is only used in the transport study, as it was necessary in forthcoming studies with enhancers. In the viability study 0.1% albumin was used, as this concentration is often sufficient to prevent adsorption of peptides.

In the construction of the Ussing chamber we have focused on reducing the chamber volume to 1 ml, in order to increase the sensitivity of the whole system and to reduce the cost of peptide. With respect to adsorption we have placed emphasis on avoiding plastic tubing and glass in the system.

In conclusion, we have found that the isolated nasal mucosa is viable for more than 10–12 h. The system may be useful for physiological and toxicological studies. Whether it is usable to screen for absorption enhancers for peptides remains to be confirmed.

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